XXXV. CATALASE ACTIVITY DURING THE DEVELOPMENT OF THE CHICK EMBRYO

By A. KLEINZELLER and HENRY WERNER

From the Cancer Research Institute, Brno, Czechoslovakia

(*Received 19 September 1938*)

The present note describes the changes in the catalase content of developing chicken embryos from the 4th to the 15th day inclusive.

The study of changes in enzyme content during development of embryo is of much interest in regard to the differentiation of metabolism. Edlbacher [1934] for instance observed a much greater concentration of arginase in young animals than in adult ones. The activity of the arginase of a chick embryo at different stages of development falls steeply from the beginning of development to the end. Edlbacher's suggestion is that arginase is concerned with a formation of arginine for the nucleoprotein of the nuclei.

Experimental methods

The chick embryo was extracted from the egg, soaked for 1 min. in distilled water and accurately weighed. The embryo was then ground with powdered sand and the product dissolved in sufficient water, to make the final strength of

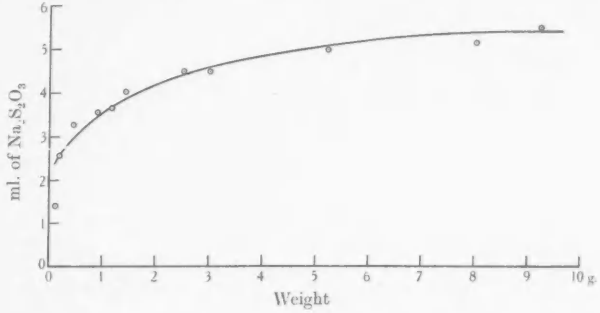


Fig. 1.

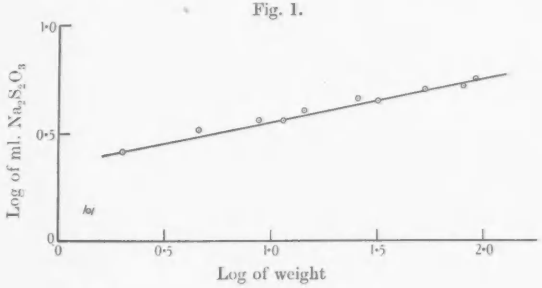


Fig. 2.

extract the same in all cases, viz. 10 mg. embryonic tissue per 1 ml. solution. The extracts were now centrifuged and 10 ml. of the fluid brought into contact with 20 ml. of 0·1 % H_2O_2. After 15 min., 5 ml. of the above were added to 5 ml. of 1 % KI and 3 ml. of 33 % H_2SO_4. The liberated I_2 was determined by titration with $N/100$ $Na_2S_2O_3$. The activity of the catalase is given by the difference between the quantity of the $Na_2S_2O_3$ employed in the control and in the experiment. The results are shown in Fig. 1. When the results are plotted logarithmically as in Fig. 2, a straight line is obtained.

If we compare our results with those of Warburg *et al.* [1924], upon the course of glycolysis during embryonic growth we find a certain relationship between the two curves. In the initial stages of the embryonic development we find on the one hand the highest values of glycolysis and on the other the lowest values of catalase activity. Towards the end of development the glycolysis decreases and the catalase rises. It is certain that the catalase does not represent the only enzyme of respiration, but it can be employed as an indicator of the changes taking place in respiratory characteristics during embryonic growth.

SUMMARY

Figures are given for the rise in catalase content during the development of the chick embryo. The activity of the catalase is inversely related to the anaerobic glycolysis of the chick embryo. High values of catalase correspond to low degrees of glycolysis and vice versa.

REFERENCES

Edlbacher (1934). Rektoratsrede Basel.
Warburg, Posener & Negelein (1924). *Biochem. Z.* **152**, 309.

XXXVI. STUDIES ON CHLOROPLASTS

I. SEPARATION OF CHLOROPLASTS, A STUDY OF FACTORS AFFECTING THEIR FLOCCULATION AND THE CALCULATION OF THE CHLOROPLAST CONTENT OF LEAF TISSUE FROM CHEMICAL ANALYSIS[1]

By ARTHUR CHARLES NEISH

From the Department of Chemistry, Macdonald College, McGill University, Quebec, Canada

(*Received 17 December 1938*)

A NUMBER of references to experiments conducted with isolated chloroplasts are to be found in the literature. Englemann [1881] detected the evolution of oxygen by isolated chloroplasts and Ewart [1896] observed that they maintained their activity for some time after removal from the cell. Chloroplasts have been isolated from *Stellaria media* by Hill [1937] and from the leaves of the tomato plant by Granick [1937]. Noack [1927] obtained disintegrated chloroplasts by fractional centrifuging of green leaves ground with $CaCO_3$ and Lubimenko [1929] employed autolysis to decompose cytoplasmic material. All of these workers had been concerned primarily with studies which required relatively small numbers of chloroplasts, and in most instances the details of the method of isolation were omitted.

The isolation of chloroplasts in large quantities would allow the study of their chemical structure and of the distribution of metabolites in the leaf. A study of photosynthesis by isolated chloroplasts could be carried out on a large scale and the problem of the photosynthesis of sugars by the plant attacked from this angle.

A procedure has been developed which gives a good yield of chloroplast substance suitable for chemical studies and also a slightly different procedure which gives morphologically intact chloroplasts.

After this work was completed Menke [1938] described the separation of the leaf tissue of *Spinacia oleracea* into four fractions, namely, cell wall, cytoplasm, water-soluble and chloroplast substances. This method is similar in principle to the one described herein, but is less convenient to use when only the chloroplast substance is desired. He does not give evidence for the chloroplasts being morphologically intact in his preparations. More recently Granick [1938] has described a similar method which gives morphologically intact chloroplasts.

Since the method of separating chloroplast granules depends on their flocculation, a study of the factors influencing the rate and degree of flocculation has been made. A knowledge of the percentage of the dry weight of leaf tissue which is contributed by chloroplasts is desirable in any study of the distribution of metabolites in leaf tissue. The chloroplast content of various leaves has been determined directly by weighing the chloroplast substance separated from a known amount of leaf tissue and indirectly by calculation from the analyses of

[1] Macdonald College Journal Series No. 108.

the chloroplast substance and corresponding leaf tissue for chlorophyll, carotene and xanthophyll which are known to be present only in the chloroplasts. The results obtained are discussed with reference to the relative ease of extraction of the plastid pigments and the condition of the plastid pigments in the chloroplasts.

Experimental

Chloroplasts exhibit osmotic properties similar to those of cells. The evidence for the existence of an osmotically active membrane surrounding the chloroplasts has been reviewed by Weier [1938]. Observations, in this connexion, have been made in order to determine the effect on the chloroplasts of the medium used in their isolation.

Chloroplasts (*Trifolium pratense*) swell and disintegrate after a few minutes in distilled water. The swollen chloroplast becomes vacuolated and appears granular in structure. These granules are discoid or spherical, green in colour and have a smooth surface. They appear to be optically homogeneous chloroplasts but are about one-sixth of the size of normal chloroplasts. The granules also swell and become vacuolated in distilled water but much more slowly than chloroplasts. They may stand in distilled water for several weeks without rupturing. When chloroplasts are isolated with distilled water the material so obtained is made up chiefly of these granules, which may therefore be regarded as intact bodies representing the chlorophyllous structure of the cell. When 0·5 *M* glucose or sucrose solutions are used instead of distilled water the chloroplasts do not all disintegrate and the preparations obtained consist of intact chloroplasts as well as free granules. There is no special advantage in using these sugar solutions when one is isolating the chloroplast substance for chemical studies, the object of which is to determine the nature of the medium in which the photosynthesis of carbohydrates occurs. The analysis of these granules, which are probably the ultimate seat of photosynthesis, should provide the desired information. A method has been evolved which enables one worker to prepare 2–3 g. (dry wt. basis) of the chloroplast substance per day.

The principles on which this method is based are as follows. (*a*) If leaf tissue is ground in a mortar, without the addition of any other solid substance to act as a grinding agent, the cells will be ruptured, freeing the chloroplasts. (*b*) Starch grains are larger and denser than chloroplasts and may be separated from chloroplasts by centrifuging the suspension at a controlled speed. Only a few of the chloroplasts will be removed by this treatment. (*c*) Chloroplast granules in suspension may be flocculated by ions such as Ca^{++} and Mg^{++} and when flocculated they settle by gravitation.

The exact details of the method vary with the material. Four species have been employed successfully, namely *Trifolium pratense* (red clover), *Elodea canadensis*, *Arctium minus* (common burdock) and *Onoclea sensibilis* (sensitive fern). Species with fibrous leaves such as *Agropyron repens* (couch grass), or mucilaginous leaves such as the young leaves of *Tilia glabra* (basswood) do not provide suitable material. In preparing the sample as much fibrous material as possible should be removed, e.g. with red clover the three leaflets are taken by breaking at the point where they join together; with *Elodea* the bushy, green tips are taken; with burdock, the large midrib is removed from the leaf and with *Onoclea* the leaflets of the fronds are broken off about half an inch from the petiole.

After washing the leaves in distilled water and squeezing by hand to remove excess water, about 20 g. of the compressed material are sliced with scissors into

an 8 in. porcelain mortar and pounded to a pulp. About 200 ml. of distilled water are added, in three portions, and the pulp ground after each addition. This gives a suspension of free chloroplast granules in the diluted cytoplasm. Most of the fibre and unground material is next removed by filtering the suspension through bolting-silk (200-mesh) and squeezing the cloth. The suspension is now centrifuged in 250 ml. cups at 2000 r.p.m. In the case of the sensitive fern it is necessary to reduce the speed of centrifuging since the chloroplasts are larger and contain starch. The purpose of this treatment is to remove the starch grains which sediment at a much lower speed than do the chloroplasts. Some of the chloroplast granules will be carried down with the starch granules. The supernatant suspensions are decanted into a 1-litre measuring cylinder until 950 ml. are obtained, 2 *M* $CaCl_2$ solution is added to the 1000 ml. mark and the contents of the cylinder mixed by inverting several times. The suspension is kept for about 30 min. when the flocculated chloroplast granules will have settled to about the 200 ml. mark. The supernatant liquid is now decanted or siphoned off; the flocculated chloroplast granules centrifuged at the same speed as before; the supernatant liquid decanted and the chloroplast granules triturated with distilled water using a glass rod with a rubber policeman. The centrifuging and washing of the chloroplast granules is repeated, usually three times, until the concentration of the flocculant is reduced to such a degree that the chloroplast granules start again to disperse. Probably only a trace of the flocculant remains at this stage. The material in the bottom of the centrifuge tube may be dried *in vacuo* at 60° for chemical analysis or redispersed if it is desired to determine enzymic activity. This material is a healthy green colour and appears to be fairly free from foreign material such as starch grains, nuclear material and cell wall fragments.

If intact chloroplasts are desired 0·5 *M* glucose can be used instead of distilled water and the chloroplasts centrifuged out at a high speed, without using a flocculating agent. This method can easily be modified to conform with the procedure of Menke [1938] for recovering cytoplasmic, water-soluble and cell wall material.

The factors influencing the rate of flocculation of the chloroplast substance in suspension

Many tests were carried out simultaneously, in all of which 10 ml. of the suspension of chloroplast substance, usually from red clover, were mixed with 10 ml. of a solution of the flocculant being tested. Unless otherwise stated the final concentration of the flocculant, after mixing, was always 0·1 *M*. The results of these experiments and the inferences drawn therefrom may be summarized as follows.

A. The rate and degree of flocculation were found to be the same with $MgCl_2$, $Mg(NO_3)_2$ and $MgSO_4$; therefore the activity was due to the cation.

B. The rate of flocculation depended on the species of cation used.

Group 1. Cations which gave a good flocculation in 1 min.

$$Ag^{+} > Pb^{++} > Fe^{++}$$

Group 2. Cations which required about 15 min. to give the same degree of flocculation as Group 1.

$$Co^{++}, Cu^{++} > Zn^{++}, Cd > Sn^{+++} > Mg^{++}, Ba^{++}, Ca^{++}, Mn^{++}$$

Group C. Cations which gave very little flocculation in 24 hr.

$$Sn^{++++} > Fe^{+++} > Al^{+++} > Na^{+}, K^{+}, NH_4^{+}$$

C. The suspensions are flocculated by hydrogen ions and the rate at which they flocculate depends, in some cases, upon the *p*H. When HCl or phosphate buffer solutions are used as flocculants the chloroplast substances of *Trifolium pratense* and *Arctium minus* show optimum flocculation at about *p*H 3·7, whereas the chloroplast substances of *Onoclea sensibilis*, *Elodea canadensis* and *Solidago* sp. show no such optimum and may be flocculated with equal facility at *p*H 5·5 and *p*H 2·0 or even when suspended in 2*N* HCl.

D. The flocculating power of a salt solution cannot be explained as being due entirely to a favourable *p*H. At *p*H 6·0 the rate of flocculation of suspensions of *Trifolium* chloroplasts decreased with decreasing concentrations of $CaCl_2$ (0·1–0·0005 *M*). However, $CaCl_2$ and $FeCl_2$ at the same concentration were much better flocculants at *p*H 3·0–3·2 than at *p*H 4·3–4·9, whereas the flocculating power of $AgNO_3$ was equally good in both ranges of *p*H.

Electrophoresis of a suspension of *Trifolium* chloroplast granules showed the chloroplast granules to be electrically charged, their mobility depending upon the *p*H. In 0·2*N* HCl the chloroplast substance was positively charged and in distilled water it was negatively charged. In these respects, then, the behaviour of *Trifolium* chloroplasts was analogous to that of protein molecules with an isoelectric point about *p*H 3·7. The resemblance of the flocculation of the chloroplast substance to the precipitation of proteins was shown by Noack [1927] who observed their "salting out" by $(NH_4)_2SO_4$. The amphoteric nature of the chloroplast substance was not, however, the same in different plants, as it was not shown by the chloroplast substance of *Onoclea sensibilis*, which, like that of *Trifolium pratense*, contains large amounts of protein.

Flocculation by salts such as $CaCl_2$ and $Mg(NO_3)_2$ was reversible. If the flocculant was washed out the chloroplast substance dispersed again and the process could be repeated. Fe^{+++} and Al^{+++} were very poor flocculants whereas Fe^{++} and Ag^{+} were excellent flocculants. The failure of trivalent ions to cause flocculation might have been due to the fact that they were present in sufficient concentration to reverse the charge. That such was not the case was indicated by the fact that negligible flocculation was obtained with varying concentrations of $FeCl_3$ (*M*/12–*M*/394) and $AlCl_3$ (*M*/20–*M*/740). It was of interest to note that the chloroplast substance prepared from red clover which was growing in the autumn and had been exposed to frost, flocculated almost as well with $AlCl_3$ as with $CaCl_2$. Silver was the only univalent ion which was a good flocculant. It was reduced by the ascorbic acid contained in the chloroplast. Presumably, therefore, the reduction of Ag^{+} removes electrons from the chloroplast substance thus reducing the negative charge and causing flocculation. The flocculating power of an ion may therefore depend on several factors such as electrical charge, age of chloroplast, solubility of a protein or lipin salt, or on some specific reaction between the ion and a constituent of the chloroplast.

The calculation of the chloroplast content of leaf tissue

The chloroplast content of leaf tissue may, theoretically, be determined in two ways: (*a*) by weighing the chloroplast substance separated as quantitatively as possible from a known amount of leaf tissue; (*b*) by analysing the chloroplasts and corresponding leaf tissue for some substance known to be found only in the chloroplasts, such as chlorophyll, carotene or xanthophyll. If, for example, 0·1 g. of leaf tissue gives a chlorophyll extract the colour of which is one-third the intensity of that derived from the same weight of chloroplast substance prepared from the same leaf tissue, then one-third of that leaf tissue is composed of chloroplasts. Obviously the first procedure will give the minimum value since

it is difficult to get all of the chloroplasts out of a given quantity of leaf tissue and since some of the chloroplast substance sediments out with the starch grains.

The values derived by different methods for the chloroplast content of the same leaf tissue are shown in Table I, and for different leaf tissues in Table II.

Table I. *Chloroplast content of red clover leaf tissue, as derived by different methods*

Method employed	Chloroplast content %
Isolation and weighing	21·7
Free chlorophyll (acetone extract)	62·1
Bound chlorophyll (acetone extract)	22·9
Carotene + xanthophyll	45·4
Carotene alone	32·2
Xanthophyll alone	50·0
Total chlorophyll (HCl treatment)	34·3

Table II. *Chloroplast content (%) of the leaf tissues of different plants, as derived by different methods*

Species	From carotene content	From chlorophyll (HCl treatment)	From xanthophyll content	From isolation and weighing
*Trifolium pratense**	32·2	34·3	50·0	21·7
*Trifolium pratense**	31·1	31·4	50·0	—
*Trifolium pratense**	26·3	25·0	41·4	—
Elodea canadensis	16·6	36·1	10·0	30·9
Onoclea sensibilis	36·9	—	37·4	13·1
Articum minus	33·9	33·3	54·1	10·7

* Each line represents a different preparation.

Values from carotene have an approximate error of ±2%; from chlorophyll ±1·5%; and from xanthophyll ±4%.

The terms "free" and "bound" chlorophyll require some explanation. A chloroplast preparation was suspended in an 85% solution of acetone in a Büchner funnel fitted with filter paper; after a few minutes suction was applied. The extraction of chlorophyll with 85% acetone was repeated until the extract was colourless, and then the residue was extracted repeatedly and in the same manner with 10% trichloroacetic acid. A yellow pigment was removed by this treatment. If the trichloroacetic acid extraction were now followed by further extractions with 85% acetone the extracts contained considerable quantities of green pigment. The pigment thus extracted showed the typical red fluorescence of chlorophyll dissolved in acetone; it was found in whole leaf tissue and the chloroplast substance and was designated as "bound" chlorophyll.

Total chlorophyll was determined by treatment with HCl as follows: 0·1 g. portions of dried and thoroughly ground tissue were weighed into 50 ml. centrifuge bottles and extracted with 10 ml. of water by heating in a boiling water bath for about 10 min., centrifuging and decanting the supernatant liquid. This removed a brown pigment from the whole leaf tissue which interfered with the determinations. After the removal of this pigment the remainder of the procedure was carried out, simultaneously and in identical manner, with an equal weight of chloroplasts and leaf tissue, as follows: 10 ml. of 6*N* HCl were added, the suspension was heated on a boiling water bath for a few min., centrifuged and the supernatant liquid containing the green-coloured products of chlorophyll hydrolysis was decanted through filter paper into a 25 ml. glass-stoppered

graduated cylinder. The extraction was repeated using 5 ml. of HCl, each time, until all the green pigment had been extracted. The combined extracts were mixed and the colour of the extract from the chloroplasts compared with that from the corresponding leaf tissue. The more intensely coloured extract was diluted until, on visual comparison, the colours of the two solutions appeared to be of equal intensity. The relative volumes were noted from which the chloroplast content of the leaf tissue was calculated.

Carotene and xanthophyll determinations were carried out by weighing 0·1 g. of the dried and ground tissue into a 50 ml. centrifuge bottle and adding 3 ml. of 30% KOH in absolute methyl alcohol and 20 ml. of light petroleum. The bottles were then stoppered and shaken for 5 min. in a mechanical shaker, centrifuged and the supernatant liquid siphoned off into separating funnels. The process was repeated, until no more pigment was extracted, using 10 ml. of light petroleum and 1 ml. of alcoholic KOH. The addition of a small amount of water was sometimes helpful. The carotene and xanthophyll contained in the combined petroleum extracts were separated by extraction with 85% methyl alcohol. The carotene content of the petroleum solution and the xanthophyll content of the methyl alcohol solution were determined by using an Evelyn photoelectric colorimeter and referring to a standard calibration curve prepared from pure β-carotene.

The results obtained from the chlorophyll determinations by HCl treatment and from the carotene determinations compare well and appear to represent the true values (see Table II). The only exception is *Elodea canadensis* where the value derived from carotene is obviously wrong, being lower than the figure obtained by isolation and weighing. The latter values are always low but not to the same extent with different species. A smaller fraction of the total number of chloroplasts is obtained in species with fibrous leaves, such as *Arctium* and *Onoclea* than in species with non-fibrous leaves such as *Elodea*. The chloroplast content of *Onoclea sensibilis* could not be estimated from chlorophyll determinations by HCl treatment because of the presence in the extract of the whole leaf tissue of a pink colour which interfered with the colorimetry.

The values shown in Tables I and II indicate the difficulties to be encountered in the extraction of the plastid pigments. If, for example, a value higher than the true value for the chloroplast content is obtained, as is the case in the calculation of the chloroplast content of the leaves of *Trifolium pratense* from xanthophyll determinations, then this means that relatively more xanthophyll has been extracted from the whole leaf than from the chloroplast substance. If the value is too low, as in the case of *Elodea canadensis*, then the reverse holds. The results in Table I show that chlorophyll and xanthophyll are extracted with difficulty from *Trifolium* chloroplast substance by 85% acetone. Carotene is apparently extracted with equal facility from the chloroplast substance and whole leaf tissue. Both carotene and xanthophyll are more easily extracted from the chloroplast substance of *Elodea* than from the whole leaf tissue, etc. Since the green chlorophyll derivatives obtained by the HCl treatment are extracted with equal ease from the chloroplast substance and whole leaf tissue in all cases, this appears to be the most dependable method to employ.

The differences in the ease of extraction of the plastid pigments from the chloroplasts as compared with the whole leaf tissue of the same species and as contrasted with other species, can apparently be explained only by assuming the presence of other concomitant substances which condition their solubility. This view was held by Willstatter. According to Lubimenko [1929] chlorophyll *a* and *b*, carotene and xanthophylls exist in the leaf in combination with protein in the

form of a single complex pigment molecule which shows slight variations in complexity in different species. We have observed that a purified chlorophyll preparation on treatment with HCl in the manner already described yields no green-coloured derivatives and that the addition of this chlorophyll preparation to chloroplasts does not increase the amount of green pigment extracted with HCl over that obtained from the chloroplasts alone. These observations are interpreted as showing that chlorophyll, as it exists in the chloroplast, perhaps in the complex pigment of Lubimenko, is decomposed by HCl treatment in a different manner from chlorophyll which has been extracted by acetone and freed from carotenoid pigments.

SUMMARY

A method which enables one worker to isolate chloroplasts from leaf tissue at the rate of 2–3 g. daily has been described.

The chloroplast substance in suspension is charged; the charge depends on the *p*H being negative above *p*H 3·7. Various cations cause the flocculation of these suspensions. This flocculation cannot be explained as a simple electrical neutralization of charges; evidently specific reactions may occur between the chloroplast substance and certain cations which are responsible for the anomalies noted. The age of the chloroplast may have some effect.

Several methods for calculating the chloroplast content of leaf tissue have been worked out and compared. These methods are based on analysis for the plastid pigments. The results obtained show that completeness of extraction of these pigments is affected by the presence of unknown substances. A simple method based on analysis for chlorophyll gives satisfactory results in most cases.

I wish to express my thanks to Dr W. D. McFarlane for the facilities extended, for helpful criticism and for assistance in preparing the manuscript.

REFERENCES

Englemann (1881). *Bot. Ztg.* **39**, 446.
Ewart (1896). *J. linn. Soc.* **31**, 364.
Granick (1937). Abstracts of 14th Annual Meeting of Am. Soc. of Plant Physiologists, 28–30 Dec.
—— (1938). *Amer. J. Bot.* **25**, 558.
Hill (1937). *Nature, Lond.*, **139**, 881.
Lubimenko (1929). Cited by J. H. Priestly, *New Phytol.* **28**, 197.
Menke (1938). *Z. Bot.* **32**, 273.
Noack (1927). *Biochem. Z.* **183**, 135.
Weier (1938). *Bot. Rev.* **4**, 475.

XXXVII. STUDIES ON CHLOROPLASTS
II. THEIR CHEMICAL COMPOSITION AND THE DISTRIBUTION OF CERTAIN METABOLITES BETWEEN THE CHLOROPLASTS AND THE REMAINDER OF THE LEAF[1]

By ARTHUR CHARLES NEISH

From the Department of Chemistry, Macdonald College, McGill University, Quebec, Canada

(*Received 17 December 1938*)

The available information concerning the chemical composition of the chloroplasts and the distribution of elements within the cell has been gained by the use of histochemical methods. The studies reported in this paper were made on the chloroplast substance isolated by the method outlined in the previous paper [1939]. Some information on the composition of the chloroplasts isolated from the leaves of two different species was first obtained. The distribution of N, P, Mg, Fe and Ca in the chloroplasts was next investigated by means of various solvents to obtain information on the nature of the combination of these elements. To determine which elements or compounds were concentrated in the chloroplasts, comparative analyses were made of chloroplast preparations and the corresponding whole leaf tissue; these comprised determinations of Ca, Fe, Cu, P, Mg, K, Na, Mn, SO_4, Cl, NH_4 salts, nitrates, ascorbic acid, catalase and carbonic anhydrase in the chloroplasts and whole leaf tissue of four different species of plants.

Experimental

Analyses were made of the whole leaf tissue and of the chloroplast substance separated from the leaf tissues of *Trifolium pratense* and *Onoclea sensibilis*. The samples were dried *in vacuo* at 60°. The protein content was estimated by determining the N content of the residue remaining after exhaustive extraction of the material with 85% acetone, absolute alcohol, anhydrous ether, 10% trichloroacetic acid and 85% acetone in the order named, and multiplying the value obtained by 6·25. The difference between the total N and the protein-N is represented as the non-protein-N. The ether extract was determined by continuous extraction of the original material in a Soxhlet apparatus with ether only. Starch was determined by the taka-diastase method of Denny [1934] and ammonia by the method of Pucher *et al.* [1935]. In determining nitrates, 0·20 g. of the material was extracted first with ether and then with boiling water; the extracts were combined and freed from ether. The aqueous extract was next made slightly alkaline and evaporated to dryness; the residue was taken up in water and brought to 200 ml. Nitrates were determined in this solution by means of the colorimetric diphenylbenzidine method of Whelan [1930].

Ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol according to McHenry & Graham [1935]. The titrations were carried out in triplicate with suspensions of freshly prepared chloroplasts or ground leaf

[1] Macdonald College Journal, Series No. 109.

tissue, in the preparation of which a few drops of 10% KCN were added to prevent oxidation of ascorbic acid. The suspensions were saturated with H_2S and kept overnight in a closed vessel. The H_2S was displaced by CO_2, the nitroprusside test being employed to determine when all the H_2S had been removed. The titration was carried out in a centrifuge tube with the least possible delay. A few ml. of 2 *M* $CaCl_2$ were added to ensure the flocculation of the suspension, more of the dye solution was added and the suspension centrifuged until the end-point of the titration was reached, which could be easily detected in the clear supernatant liquid. The first titration gives an approximate value; the second and third titrations can be conducted more accurately.

Carotenoid pigments were determined as previously described [1939]. P was determined according to Dyer & Wrenshall [1938]; Fe and Cu by the methods of Parker & Griffin [1939]; Ca by the volumetric micromethod of the A.O.A.C. [1935]; Mg by the hydroxyquinoline method of Greenberg *et al.* [1935]; Na by the method of Salit [1932]; K by the volumetric cobaltinitrite method of Volk & Truog [1934]; Mn by the colorimetric bismuthate method of the A.O.A.C. [1935]; SO_4 by the volumetric benzidine method of Cope [1931] and Cl by the method of Cattle [1935].

The catalase and carbonic anhydrase activities of suspensions of the chloroplast substance and ground whole leaf tissue in distilled water were determined with a differential manometer. It was necessary to use the thermostat at a temperature of 27° because of the high atmospheric temperature prevailing at the time. The amount of gas evolved was plotted against time for suspensions of known dilution and in this way the time was found at which the amount of gas evolved is a measure of the enzymic activity.

In the fractionation of the chloroplasts a 2 g. sample of the moisture-free, finely pulverized chloroplasts was employed in each case. The extractions with anhydrous solvents were made in a Soxhlet extractor, but with the other solvents repeated extractions were carried out in a Büchner funnel fitted with filter paper.

Table I. *Analysis of the chloroplast substance of* Trifolium pratense *and* Onoclea sensibilis

Results expressed as % of the moisture-free substance

	Trifolium pratense	*Onoclea sensibilis*
Total N	9·69	5·90
NH_4-N	0·80	0·12
NO_3-N	0·011	0·006
Non-protein-N	1·66	0·87
Protein-N	8·02	5·05
Total protein	50·11	31·51
Ether extract	21·75	7·21
Starch	Nil	8·43
Ascorbic acid	2·53	0·56
Carotene	0·19	0·10
Xanthophylls	0·24	0·15
Total ash	3·02	4·11
Mg	0·166 : 0·164	0·113 : 0·164
Cu	0·008	0·017
Fe	0·092	0·074
Ca	0·170	0·220
P	0·219 : 0·741	0·326 : 0·254
K	Nil	Nil
Na	0·039	0·939
Mn	Trace	Trace
SO_4	0·894	0·431
Cl	Nil	0·074

Results and discussion

1. *Composition of chloroplasts*

The results are shown in Table I. The high protein and lipoid contents of the chloroplasts, particularly of *Trifolium pratense*, are noteworthy. These results agree as well as could be expected with those of Menke [1938] who records 8·38 % N and 30·9 % ether extract for the chloroplasts of *Spinacia oleracea*. The chloroplasts of *Onoclea sensibilis* are different from those of the other two species; they are considerably larger and appear to store more starch than those of clover, which accounts, in part at least, for the lower lipoid and protein contents. It will also be noted that the P content varied markedly in two preparations from the leaves of the same species cut at different stages of growth.

2. *Fractionation of chloroplasts*

In Table II are shown the scheme of extraction followed and the results obtained. When the initial extraction was made with 85 % acetone more material was dissolved than when it was made with anhydrous ether (Table II B). Furthermore, extraction with 85 % acetone left only a small fraction which was extractable by alcohol and ether, so that most of the lipoid material of the chloroplast was extracted by 85 % acetone. An appreciable amount of P was extracted by anhydrous ether but when the material was first extracted with 85 % acetone the amount of P extracted by ether was considerably reduced. It would appear, therefore, that the chloroplasts contain phospholipins which are appreciably soluble in 85 % acetone. Granick [1937] found that 40 % of the total lipoid-P of the tomato leaf was located in the chloroplasts. Most of the P in clover chloroplasts was extracted by trichloroacetic acid, whereas the P in the chloroplasts of *Onoclea sensibilis* was found to be more difficult to extract.

Nearly all of the Mg contained in the chloroplasts was extracted by trichloroacetic acid, and when compared with this fraction the amount of Mg present in the chlorophyll fraction was negligible. Ca was also nearly all removed by the trichloroacetic acid. This is in accord with the findings of Terlikowski and Sozonski [1937] and Kostychev & Berg [1929], that extraction of leaf tissue with dilute HCl acid effects almost complete removal of the Ca and Mg.

A considerable proportion of the total Fe was extracted by trichloroacetic acid and an appreciable amount was also dissolved by previous extraction with ether, which is to be expected from the known solubility of inorganic salts of Fe in ether. Even after extraction with trichloroacetic acid, Fe was found in a subsequent 85 % acetone extract. The Fe contained in the chloroplasts of *Onoclea sensibilis* seems to be in a form readily soluble in acetone which may account for the comparatively small amount contained in the trichloroacetic acid fraction. The nature of the combination of Fe in the chloroplast is discussed later. The ionic state of the Ca, Mg, P and Fe in the trichloroacetic acid extracts was shown by the fact that the amounts of these elements, as obtained by direct analysis of the trichloroacetic acid solution, were nearly equal to the amounts obtained after digestion with conc. H_2SO_4 plus Na_2SO_4 and HgO.

Most of the N remains in the final residue and is probably all protein-N. Small amounts are extracted by trichloroacetic acid or ether, that dissolved by the latter probably being contributed by lecithin. Granick [1938] found that 80 % of the total N in chloroplasts was protein-N. This agrees with the values reported here.

Table II. *Showing the analysis of various fractions separated from the chloroplasts of* Trifolium pratense *and* Onoclea sensibilis

Results expressed as % of the total amount in the original material

A. Dry, powdered, chloroplast substance extracted with anhydrous ether

Residue
(Extracted with absolute alcohol, followed by asolute acetone)

Residue
(Extracted with 80% acetone, followed by extraction with 10% trichloroacetic acid)

	Ether extract		Acetone extract		Trichloroacetic acid extract	
	Trifolium	*Onoclea*	*Trifolium*	*Onoclea*	*Trifolium*	*Onoclea*
Dry wt.	21·75	7·21	0·55	0·90	3·35	0·42
N	2·26	0·73	0·18	0·93	67·97	14·12
P	3·10	1·13	*	0·015	90·85	78·76
Mg	*	9·74	*	*	66·96	54·21
Fe	6·11	*	3·49	6·52	92·12	103·64

B. Dry, powdered, chloroplast substance extracted with 85% acetone

Soluble in 85% acetone
(Residue extracted with light petroleum)

Residue
(Extracted with absolute alcohol and anhydrous ether)

	Extract		Extract	
	Trifolium	*Onoclea*	*Trifolium*	*Onoclea*
Dry wt.	25·14	12·24	3·75	1·53
N	5·86	1·37	0·32	0·32
P	1·42	6·90	0·23	0·64
Mg	*	*	*	*
Fe	*	*	1·53	2·44

Residue
(Extracted with 10% trichloroacetic acid)

Residue
(Extracted with 80% acetone)

	Extract		Extract (bound chlorophyll)		Residue	
	Trifolium	*Onoclea*	*Trifolium*	*Onoclea*	*Trifolium*	*Onoclea*
N	3·18	0·32	3·03	0·63	82·73	85·51
P	55·43	19·31	3·88	11·57	—	—
Mg	87·80	61·95	*	*	—	—
Fe	47·76	26·49	2·29	7·74	—	—
Ca	92·12	83·62	—	—	—	—

* If present, the amount found was too small to be determined by the methods employed.

3. *Comparative analysis of chloroplasts and whole leaf tissue*

The results of the analysis of the whole leaf tissue of different species of plants and of the chloroplast substance separated from the same leaf tissue are shown in Tables III, IV and VII. When reported as % of the total ash

Table III. *Showing the comparative compositions of the ash of chloroplasts and whole leaf tissue*

Species	Total ash (% dry wt.)	% of the total ash									
		Fe	Ca	Mg	P	Mn	K	Na	Cu	SO_4	Cl
Trifolium pratense:											
Chloroplast substance	8·04	0·44	6·15	1·99	9·20	—	*	0·48	0·101	11·11	*
Whole leaf	9·91	0·27	20·87	3·08	3·84	—	20·01	3·21	0·042	7·31	1·11
Onoclea sensibilis:											
Chloroplast substance	5·23	1·08	6·33	3·04	4·90	—	*	0·74	0·332	8·23	2·12
Whole leaf	9·75	0·44	13·31	5·12	3·80	0·06	21·01	2·14	0·077	10·58	5·63
Elodea canadensis:											
Chloroplast substance	9·74	2·56	—	2·07	4·01	0·24	*	2·57	0·165	6·89	—
Whole leaf	12·89	1·14	—	3·41	3·56	0·52	25·28	5·16	0·068	6·21	—
Arctium minus:											
Chloroplast substance	5·32	1·18	9·62	3·00	6·39	—	*	0·39	0·160	8·09	0·74
Whole leaf	11·75	0·59	14·02	4·86	2·64	—	21·68	3·35	0·031	5·21	3·54

* Presence doubtful—not detectable by the methods used.

Table IV. *Showing the distribution of ammonia, nitrates, sulphates and ascorbic acid in leaf tissue*

Results are expressed as % of the moisture-free substance

Species	Ammonia (% NH_4-N)			Nitrates (% NO_3-N)		
	Chloroplasts	Whole leaf	Non-chloroplast fraction	Chloroplasts	Whole leaf	Non-chloroplast fraction
Trifolium pratense	0·794	0·335	0·165	0·0110	0·0062	0·0045
Onoclea sensibilis	0·125	0·088	0·066	0·0060	0·0034	0·0051
Elodea canadensis	0·234	0·110	0·033	0·0006	0·0044	0·0073
Arctium minus	0·217	0·172	0·149	0·0032	0·0156	0·0208

	Sulphates			Ascorbic acid		
	Chloroplasts	Whole leaf	Non-chloroplast fraction	Chloroplasts	Whole leaf	Non-chloroplast fraction
Trifolium pratense	0·89	0·66	0·57	2·529	2·557	2·566
Onoclea sensibilis	0·43	1·03	1·38	0·566	0·285	0·126
Elodea canadensis	0·67	0·80	0·88	0·653	0·433	0·325
Arctium minus	0·43	0·61	0·70	0·497	0·677	0·770

(Table III), or as % of the total amount in the whole leaf which is contained in the chloroplast fraction (Table V), the values show which elements or compounds are concentrated in the chloroplasts relative to the other fractions. The whole leaf material is always higher in total ash content than the chloroplast substance. From these results the following observations may be made.

A. *Fe and Cu* are concentrated in the chloroplasts, Cu showing an even greater tendency to collect in the chloroplasts than Fe. The fact that Fe and Cu show the same localization supports the view that some oxidative reactions are centred in the chloroplasts which particularly require their catalytic action.

Table V. *Showing the distribution of elements between the chloroplasts and the remainder of the leaf tissue*

Results are expressed as % of the moisture-free substance

		Trifolium pratense	*Onoclea sensibilis*	*Elodea canadensis*	*Arctium minus*
% of chloroplasts in the leaf tissue		27	37	40	33
Fe:	Whole leaf	0·025	0·043	0·147	0·070
	Chloroplast substance	0·035	0·057	0·249	0·063
	Non-chloroplast fraction	0·022	0·035	0·092	0·077
Ca:	Whole leaf	1·91	1·43	—	2·45
	Chloroplast substance	0·17	0·22	—	0·72
	Non-chloroplast fraction	2·55	2·14	—	3·29
Mg:	Whole leaf	0·28	0·50	0·44	0·57
	Chloroplast substance	0·16	0·16	0·20	0·12
	Non-chloroplast fraction	0·33	0·70	0·60	0·78
P:	Whole leaf	0·35	0·37	0·46	0·31
	Chloroplast substance	0·74	0·26	0·39	0·34
	Non-chloroplast fraction	0·21	0·44	0·50	0·29
K:	Whole leaf	1·82	2·05	3·26	2·54
	Chloroplast substance	*	*	*	*
	Non-chloroplast tissue	2·49	3·25	5·43	3·79
Na:	Whole leaf	0·29	0·21	0·67	0·39
	Chloroplast substance	0·04	0·04	0·25	0·02
	Non-chloroplast tissue	0·38	0·32	0·95	0·07
Cu:	Whole leaf	0·0038	0·0025	0·0087	0·0038
	Chloroplast substance	0·0081	0·0174	0·0160	0·0085
	Non-chloroplast fraction	0·0022	0·0017	0·0038	0·0009
Cl:	Whole leaf	0·10	0·61	—	0·62
	Chloroplast substance	*	0·07	—	0·05
	Non-chloroplast fraction	0·14	0·94	—	0·90
Mn:	Whole leaf	Trace	0·01	0·07	Trace
	Chloroplast substance	Trace	Trace	0·02	Trace
	Non-chloroplast fraction	—	—	0·10	—

* Presence doubtful—not detected by methods used.

Determinations of inorganic Fe and Cu showed that whilst most of the Fe in the chloroplasts is present in the inorganic form there is an appreciable amount which may be in organic combination, and that Cu is present in the chloroplasts largely in organic combination (Table VI).

Table VI. *Nature of the combination of Fe and Cu in the chloroplasts and whole leaf tissue of* Trifolium pratense

Fe (mg. per g. dry tissue):	Chloroplasts	Whole leaf
Total	0·772	0·479
In 10% acetic acid extract	0·572	0·292
Inorganic Fe (% of total Fe)	74·1	61·0

% of the Fe in the whole leaf which is contained in the chloroplast fraction: (*a*) inorganic, 51·7%; (*b*) organic, 57·5%.

Cu (mg. per g. dry tissue):		
Total	0·081	0·038
In 10% acetic acid extract	Nil	0·004
Inorganic Cu (% of total Cu)	—	10·5

% of the total Cu in the whole leaf which is contained in the chloroplast fraction: 74·6%.

B. *Ca and Mg* show a similar localization, being found mainly in the cytoplasm, although the chloroplasts do contain appreciable amounts of these elements. This distribution is interesting from the viewpoint of Laville [1933] who believes that Mg antagonizes Fe by its antioxidant activity; the two elements show opposite localizations in the leaf thus allowing each other greater freedom of action.

C. *P*, relative to the other constituents of the ash, is concentrated in the chloroplasts; the degree of concentration, however, is variable. Granick [1937] has recorded that 20 % of the total P and 40 % of the lipoid P in tomato leaves is contained in the chloroplasts. This does not show an accumulation of P in the chloroplasts unless the chloroplast content of this leaf is very small. That the P content of the chloroplasts of *Trifolium pratense* may vary widely is shown from the two results given in Table I, which represent the P contents of the chloroplasts of the young growing plant and of the flowering plant respectively. According to Seissl [1909] the P content of the leaves is at a maximum during the period of greatest CO_2 assimilation.

D. *Na and K* are concentrated outside of the chloroplast; K is probably absent from the chloroplast. These observations are in agreement with the findings of Weevers [1913] and Lloyd [1925], by histochemical tests, that K was localized in the vacuoles and of Funcoka [1921] that Na, by similar tests, is localized in the cell plasma.

E. *Mn* appears to be present in both the chloroplasts and non-chloroplast material of the leaf and particularly in the latter. In some cases there is only a trace of Mn present.

F. *SO_4 and Cl* show a variable distribution in the leaves of different species. SO_4 in particular shows no evidence of a definite localization, whereas Cl appears to be concentrated outside the chloroplast, presumably as a result of the high concentration of potassium in the cell sap (see Table IV).

G. *NH_4 salts* appear to be more highly concentrated in the chloroplasts, whereas nitrates, like sulphates, do not show any definite plan of localization.

H. *Ascorbic acid* appears to be present in appreciable quantities both in the chloroplasts and in the other parts of the cell. This is in accord with the findings, by histochemical tests of Giroud *et al.* [1934; 1935] and of Dischendorfer [1936] who conclude further, that there is a positive correlation between the amount of chlorophyll and ascorbic acid present and that the latter is bound in the chloroplasts.

I. *Catalase and carbonic anhydrase* are found in the chloroplasts, the former being highly concentrated in the chloroplasts, in all of the species. Carbonic anhydrase is also present in the other parts of the leaf in amounts varying with the species. The values for the whole leaf tissue are a little too low from the fact that it was difficult to rupture all the cells by grinding. This explains why the values in Table VII show a higher localization of catalase in the chloroplasts than is theoretically possible. However, at least 80–90 % of the cells should be ruptured since 10 g. of the material were ground as thoroughly as possible in a large porcelain mortar. Von Euler and his associates [1929; 1930] have found a relationship between the chlorophyll content and catalase activity, and between the number of chloroplasts present and catalase activity, which appeared to be hereditary.

It is interesting to note that Fe, Cu and catalase, which are concentrated in the chloroplasts, are oxidative catalysts. This would indicate that active respiratory processes occur in the chloroplasts.

Table VII. *Showing the distribution of carbonic anhydrase and catalase in leaf tissue*

	Trifolium pratense		*Arctium minus*		*Onoclea sensibilis*	
	Carbonic anhydrase	Catalase	Carbonic anhydrase	Catalase	Carbonic anhydrase	Catalase
Activity of chloroplast* substance	13·89	7·06	57·90	12·12	50·04	13·63
Activity of whole leaf*	15·37	1·78	24·84	3·83	38·22	4·78
Activity of whole leaf due to chloroplasts (calculated)	3·75	1·90	19·30	4·30	18·51	5·04
% of total activity of leaf which is due to chloroplasts	24·39	106·74	77·69	112·27	48·43	105·43

* Carbonic anhydrase activities are represented in μl. CO_2 released per mg. of dry tissue in 30 sec. at 27°.

Catalase activities are represented in μl. O_2 released from 0·01 *M* H_2O_2 per mg. of dry tissue in 2 min. at 27°.

Summary

Chloroplasts consist chiefly of protein and lipins. They contain a relatively high percentage of lipins as compared with the rest of the cell. Nearly all the lipin fraction may be extracted with 85% acetone.

Cu, Fe, P and NH_4 salts are concentrated to a certain extent in the chloroplasts. Ca, Mg, Mn, Na, K and Cl show an opposite localization in the cell. SO_4 and NO_3 do not follow any general rule.

The Cu in chloroplasts appears to exist chiefly in organic combination. Part of the Fe and P is also combined organically but Ca and Mg are present chiefly in the inorganic state.

Most of the catalase in the leaf cells is found in the chloroplasts. Carbonic anhydrase and ascorbic acid are found in appreciable quantities both in the chloroplasts and in other parts of the cells.

I wish to express my thanks to Prof. W. D. MacFarlane for helpful criticism and assistance in preparing the manuscript and to Mr F. P. Griffin who carried out the determinations of iron and copper.

REFERENCES

Assoc. of Official Agric. Chem. (1935). Methods of Analysis, 4th ed. p. 123.

Cattle (1935). *New Phytol.* **34**, 151.

Cope (1931). *Biochem. J.* **25**, 1183.

Denny (1934). *Contr. Boyce Thompson Inst.* **6**, 129.

Dyer & Wrenshall (1938). *Canad. J. Res.* B, **16**, 97.

Dischendorfer (1936). *Protoplasma*, **28**, 516.

von Euler, Steffenberg & Hellström (1929). *Hoppe-Seyl. Z.* **183**, 113.

—— —— Runehjelm (1929). *Hoppe-Seyl. Z.* **185**, 74.

—— —— —— (1930). *Ark. Kemi Min. Geol.* **10** A, 1.

Funcoka (1921). *Jap. Med. Lt.* **6**, 2; *Kyoto Igaku Zasshi*, **15**, 85.

Giroud, Ratesmamanga & Leblond (1934). *C.R. Soc. Biol., Paris*, **117**, 612.

—— —— —— (1935). *Bull. Soc. Chim. biol., Paris*, **17**, 232.

Granick (1937). Abstracts of 14th Meeting of Am. Soc. of Plant Physiol. 28–30 Dec.

—— (1938). *Amer. J. Bot.* **25**, 561.

Greenberg, Anderson & Tufts (1935). *J. biol. Chem.* **111**, 561.

Kostychev & Berg (1929). *Z. wiss. Biol.* **8**, 56.
Laville (1933). *Néoplasmes*, **12**, 14.
Lloyd (1925). *Flora*, **118**, 369.
McHenry & Graham (1935). *Biochem. J.* **29**, 2013.
Menke (1938). *Z. Bot.* **32**, 273.
Neish (1939). *Biochem. J.* **33**, 293.
Parker & Griffin (1939). *Canad. J. Res.* B (in the Press).
Puhcer, Vickery & Leavenworth (1935). *Industr. Engng Chem.* **7**, 152.
Salit (1932). *J. biol. Chem.* **96**, 654.
Seissl (1909). *Z. Endw. Vers. Wes. Oesten*, **12**, 157.
Terlikowski & Sozonski (1937). *Bodekunde Pflanzenernährung*, **2**, 252.
Volk & Truog (1934). *J. Amer. Soc. Agron.* **26**, 537.
Weevers (1913). *Rec. Trav. bot. néerland.* **8**, 289; *Zbl. Biochem. Biophys.* **13**, 829.
Whelan (1930). *J. biol. Chem.* **86**, 189.

XXXVIII. INTERACTION OF VITAMIN C AND TISSUE PHOSPHATASES

By K. V. GIRI[1]

From the Nutrition Research Laboratories, I.R.F.A., Coonoor, South India

(*Received 4 October 1938*)

In previous publications [Giri, 1938, 1 and 2] the results of experiments on the interaction of plant phosphatases, vitamin C and glutathione were reported. A similar investigation on animal tissue phosphatases has been undertaken with a view to completing a general survey of the effect of vitamin C upon the activity of phosphatases of both plant and animal origin.

Experimental

Preparation of material. The tissues used in the present investigation were those of sheep kidney, liver and brain. Dry powders were prepared from the tissues by treatment with acetone and ether as described by Giri & Datta [1936]. Active extracts were obtained from the acetone-treated tissue materials, as described below.

The method of determining the activity of the phosphatase and the other procedures were similar to those already described [Giri, 1938, 2].

I. Kidney phosphatase

Influence of vitamin C on the activity of kidney phosphatase. 10 g. acetone-treated kidney powder were extracted with 100 ml. water, saturated with

Table I. *Influence of vitamin C on the activity of kidney phosphatases without purification by dialysis*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*p*H 8·9) (for alkaline phosphatase) and 10 ml. *M*/5 acetate buffer (*p*H 5·0); 10 ml. 2% sodium-β-glycerophosphate (Merck); 4 ml. 0·1 *M* $MgCl_2$, $6H_2O$ (only for alkaline phosphatase); 5 ml. enzyme extract. Total volume, 40 ml.; vitamin C, 5 mg.; Cu as $CuSO_4$, $5H_2O$, 0·062 mg.

	Activity in mg. in 10 ml. reaction mixture	
	Alkaline phosphatase	Acid phosphatase
Time of hydrolysis in min. ...	60	120
Crude phosphatase	0·642	0·130
Crude phosphatase + vitamin C	0·642	0·130
Crude phosphatase + vitamin C + Cu	0·620	0·130
Crude phosphatase + Cu	0·642	0·130

Oxidation of vitamin C	mg. of vitamin C in the total volume of reaction mixture	
Crude phosphatase + vitamin C	4·6	4·4
Crude phosphatase + vitamin C + Cu	2·0	3·5

[1] Lady Tata Memorial Scholar.

toluene for 24 hr. and filtered. A portion of the filtrate (30 ml.) was dialysed for 2 days in collodion bags and centrifuged to remove the suspended impurities.

The results show that the vitamin C, Cu and the vitamin C-Cu complex have no influence on the activity of the alkaline and acid phosphatases of kidney when crude aqueous extracts without purification by dialysis are used (Table I).

Table II A. *Influence of vitamin C on the activity of kidney phosphatase purified by dialysis*

Reaction mixture. 10 ml. glycine buffer $M/5$ (pH 8·9) for alkaline phosphatase and 10 ml. acetate buffer $M/5$ (pH 5·0) for acid phosphatase; 10 ml. 2% Na-β-glycerophosphate; 4 ml. enzyme extract; total volume, 40 ml.; vitamin C, 5 mg.; Cu as $CuSO_4, 5H_2O$ (0·062 mg.).

Alkaline phosphatase

	Activity in mg. P in 10 ml. reaction mixture		
Time of hydrolysis in min. ...	15	30	60
1. Purified phosphatase	0·470	0·800	1·50
2. Purified phosphatase + vitamin C	0·470	0·800	1·50
3. Purified phosphatase + vitamin C + Cu	0·300	0·500	1·08
4. Purified phosphatase + Cu	0·470	0·800	1·50

Oxidation of vitamin C

	mg. vitamin C in the total volume of reaction mixture			
Time in min. ...	0	15	30	60
2. Purified phosphatase + vitamin C	5·0	4·9	4·8	4·5
3. Purified phosphatase + vitamin C + Cu	5·0	2·8	2·4	1·3

Table II B.

Acid phosphatase

	Activity in mg. P in 10 ml. reaction mixture	
Time of hydrolysis in min. ...	60	120
1. Purified phosphatase	0·083	0·154
2. Purified phosphatase + vitamin C	0·083	0·150
3. Purified phosphatase + vitamin C + Cu	0·066	0·100
4. Purified phosphatase + Cu	0·083	0·150

Oxidation of vitamin C

	mg. vitamin C in the total volume of reaction mixture			
Time in min. ...	0	30	60	120
1. Purified phosphatase + vitamin C	5·0	4·9	4·7	4·7
3. Purified phosphatase + vitamin C + Cu	5·0	4·8	2·6	1·3

It can be seen from the figures in Tables II A and B, that the vitamin C-Cu complex inhibits the activity of the purified phosphatase, while vitamin C or copper alone has no influence. Thus the behaviour of the phosphatase present in dialysed aqueous extracts of kidney towards the vitamin C-Cu complex is different from that of the enzyme present in crude aqueous extracts of the tissue. Some factors present in the latter appear to interfere with the inhibition of the activity of the phosphatase by the vitamin C-Cu complex. In the light of the previous observations made on the influence of vitamin C on plant phosphatases, it was thought that the factors which interfere with the inhibition of the activity of the phosphatase by the vitamin C-Cu complex, protect the vitamin against oxidation, thereby preventing the inhibition. The following experiment

was therefore undertaken with a view to finding out whether the protective action of kidney extracts against the oxidation of the vitamin is responsible for the differences observed in the behaviour of crude aqueous extracts and dialysed extracts of kidney towards the vitamin C-Cu complex (Table III).

Table III. *Protective action of dialysed and undialysed kidney extracts against the oxidation of vitamin C*

Reaction mixture. 20 ml. acetate buffer *M*/5 (*p*H 5·0); 5 ml. extract; 5 mg. vitamin C; Cu as $CuSO_4, 5H_2O$ (0·062 mg.); total volume, 40 ml.

	mg. vitamin C in the total volume of reaction mixture			
Time in min. ...	0	15	30	60
1. Vitamin C	5·0	3·4	3·0	2·3
2. Vitamin C + dialysed extract	5·0	3·8	3·4	3·0
3. Vitamin C + undialysed extract	5·0	5·0	5·0	5·0
4. Vitamin C + Cu	5·0	2·7	2·5	1·4
5. Vitamin C + Cu + dialysed extract	5·0	2·5	2·4	1·0
6. Vitamin C + Cu + undialysed extract	5·0	4·5	4·1	3·6

It is seen from these results that undialysed extracts contain some substances which protect the vitamin against catalytic oxidation by Cu, while the dialysed extract does not exert any such protective action, although a slight protection in the absence of Cu is noticed. Further, the dialysed extracts contain certain substances which accelerate the oxidation of vitamin C by Cu. Such constituents, which are probably colloidal in nature, are found to be present in the dialysed extracts of acetone-treated liver and brain. Further investigation of the nature of the constituents and their relation to the oxidation of the vitamin is in progress.

The results so far obtained on the influence of the vitamin C-Cu complex on the activity of kidney phosphatase show that the activity is in some way related to the oxidation of the vitamin.

II. Liver phosphatase

The influence of vitamin C on the activity of liver phosphatase

Preparation of active extract. 20 g. acetone-treated liver powder were extracted with 100 ml. water saturated with toluene for 48 hr. at room temperature and filtered. The filtrate, which was coloured yellow, was used directly. The dialysed extract was prepared by dialysing the crude aqueous extract (60 ml.) for 3 days and centrifuging to remove the suspended impurities.

The results are similar to those obtained with kidney phosphatase. The liver phosphatase activity is inhibited by the vitamin C-Cu complex when dialysed extracts are used as the source of the enzyme, while the activity of the phosphatase present in crude aqueous extracts is not affected by vitamin C-Cu complex. Neither vitamin C nor copper alone has any influence on the activity (Table IV).

Protective action of liver extracts against the oxidation of vitamin C. Table V shows the difference in the protective properties of the dialysed and undialysed extracts of acetone-treated liver powder.

It can be seen from the results that undialysed extracts of liver protect the vitamin against catalytic oxidation, while the dialysed extracts lack such protective properties. The difference in behaviour of liver phosphatase contained in dialysed and undialysed extracts towards the vitamin C-Cu complex

Table IV. *Influence of vitamin C on the phosphatase activity of dialysed and undialysed liver extracts*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*p*H 8·9); 10 ml. 2% Na-β-glycerophosphate; 4 ml. 0·1 *M* $MgCl_2$, $6H_2O$ (only for alkaline phosphatase); 5 ml. enzyme extract; total volume, 40 ml. Vitamin C, 5 mg.; Cu as $CuSO_4$, $5H_2O$ (0·062 mg.). Temperature, 35 ±0·1°.

	Activity in mg. P in 10 ml. reaction mixture		
	Undialysed extract	Dialysed extract	
Time of hydrolysis ...	1 hr.	3 hr.	5 hr.
1. Phosphatase	0·160	0·690	1·00
2. Phosphatase + vitamin C	0·160	0·680	0·950
3. Phosphatase + vitamin C + Cu	0·160	0·520	0·714
4. Phosphatase + Cu	0·160	0·690	1·00

Oxidation of vitamin C

	mg. vitamin C in the total of reaction mixture						
	Undialysed extract			Dialysed extract			
Time in min. ...	0	30	60	0	30	60	120
2. Phosphatase + vitamin C	5·0	4·5	4·1	5·0	4·6	4·3	3·3
3. Phosphatase + vitamin C + Cu	5·0	3·8	3·3	5·0	2·2	1·5	0·2

Table V. *Protective action of dialysed and undialysed liver extracts against the oxidation of vitamin C*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*p*H 8·9); Cu as $CuSO_4$, $5H_2O$ (0·062 mg.); 5 ml. 0·100% vitamin C; 5 ml. liver extract; total volume, 40 ml. Temperature, 35 ±0·1°.

	mg. vitamin C in total volume of reaction mixture					
	Undialysed liver extract			Dialysed liver extract		
Time in min. ...	0	30	60	0	30	60
1. Vitamin C	5·0	4·3	4·0	5·0	4·4	3·8
2. Vitamin C + Cu	5·0	2·2	1·6	5·0	2·1	1·4
3. Vitamin C + liver extract	5·0	4·8	4·6	5·0	4·4	4·0
4. Vitamin C + Cu + liver extract	5·0	4·0	3·4	5·0	1·7	0·8

is due to the presence of certain substances in the undialysed extract, which protect the vitamin against catalytic oxidation, so that the activity of the phosphatase is unaffected in the presence of vitamin C and copper. When these substances are removed by dialysis, the extract loses its protective properties, and the phosphatase is inhibited by the vitamin C-Cu complex because under such conditions the vitamin is oxidized. Further, the results show that, as in the case of kidney extracts, the dialysed extracts of liver contain some factors which accelerate the oxidation of vitamin C by copper.

The influence of glutathione (GSH) on the activities of the alkaline and acid phosphatases of kidney and liver in the presence and absence of vitamin C-Cu complex

The results of experiments on the effect of glutathione on the alkaline and acid phosphatases of kidney and liver in the presence and absence of the vitamin C-Cu complex are presented in Table VI.

The results show that glutathione (GSH) protects the acid phosphatase of kidney from inhibition by the vitamin C-Cu complex, while the inhibition of the activity of the alkaline phosphatases of kidney and liver is not greatly

Table VI. *Effect of glutathione on the activities of the alkaline and acid phosphatases of kidney and liver in the presence and absence of vitamin C-Cu complex.*

Reaction mixture. (Kidney phosphatases): 20 ml. glycine buffer *M*/5 (*p*H 8·9), for alkaline phosphatase and 20 ml. acetate buffer *M*/5 (*p*H 5·0) for acid phosphatase; 10 ml. 2% Na-β-glycerophosphate; 4 ml. $MgCl_2$, $6H_2O$, 0·1 *M* (only for alkaline phosphatase); 5 ml. enzyme (dialysed extract); vitamin C, 5 mg.; glutathione, 5 mg.; Cu as $CuSO_4$, $5H_2O$ (0·062 mg.); total volume, 50 ml.

(Liver phosphatase): 10 ml. glycine buffer *M*/5 (*p*H 8·9); 10 ml. 2% Na-β-glycerophosphate; 4 ml. 0·1 *M* $MgCl_2$, $6H_2O$; 5 ml. enzyme (dialysed extract); vitamin C, 5 mg.; Cu as $CuSO_4$, $5H_2O$ (0·062 mg.); glutathione, 5 mg.; total volume, 40 ml. Temperature, 35 ± 0·1°.

	Activity in mg. P in 10 ml. reaction mixture				
	Kidney alkaline phosphatase		Kidney acid phosphatase	Liver alkaline phosphatase	
	0·5 hr.	1 hr.	12 hr.	2 hr.	4 hr.
1. Purified phosphatase	0·400	0·640	0·200	0·457	0·800
2. Purified phosphatase + vitamin C	0·400	0·640	0·200	0·450	0·789
3. Purified phosphatase + vitamin C + Cu	0·310	0·490	0·040	0·333	0·580
4. Purified phosphatase + vitamin C + Cu + glutathione (GSH)	0·310	0·500	0·200	0·350	0·640
5. Purified phosphatase + glutathione (GSH)	0·400	0·571	0·182	0·444	0·762

Oxidation of vitamin C

	Vitamin C in mg. in the total volume of reaction mixture								
	Kidney alkaline phosphatase			Kidney acid phosphatase			Liver alkaline phosphatase		
Time in min. ...	0	30	60	0	30	60	0	30	60
2. Purified phosphatase + vitamin C	5·0	3·6	3·0	5·0	4·8	4·8	5·0	4·5	4·1
3. Purified phosphatase + vitamin C + Cu	5·0	1·4	0·4	5·0	2·2	1·3	5·0	2·2	1·4
4. Purified phosphatase + vitamin C + Cu + glutathione	5·0	3·0	1·4	5·0	4·9	4·9	5·0	4·4	2·9

influenced in its presence. This difference in behaviour of glutathione is perhaps due to the fact that the vitamin is protected completely from oxidation at *p*H 5·0, which is the optimum for the acid phosphatase, while at *p*H 8·9, which is the optimum for the alkaline phosphatase, it has very little protective action. It is found that glutathione itself has a slight inhibiting action on the activity of the phosphatase.

III. Brain phosphatase

The influence of vitamin C on the activity of brain phosphatase

Preparation of extract. Extract No. 1. 20 g. acetone-treated brain powder were extracted with 100 ml. water saturated with toluene for 48 hr. at room temperature and filtered.

Extract No. 2. Purified by isoelectric precipitation of the inert material at *p*H 4·8. The extract was prepared by adding an equal volume of *M*/5 acetate buffer *p*H 4·8 to the aqueous extract, and centrifuging to remove the suspended impurities.

Extract No. 3. Prepared by dialysing the crude aqueous extract of acetone-treated brain.

Table VII. *Influence of vitamin C on the phosphatase activities of dialysed and undialysed extracts of brain*

Reaction mixture. The composition was the same as that of the mixture used for kidney and liver phosphatases.

	Activity in mg. P in 10 ml. reaction mixture after 4 hr. hydrolysis	
	Undialysed extract	Dialysed extract
1. Phosphatase	0·158	0·110
2. Phosphatase + vitamin C	0·158	0·110
3. Phosphatase + vitamin C + Cu	0·090	0·060
4. Phosphatase + Cu	0·158	0·110

Oxidation of vitamin C

	mg. vitamin C in the total volume of reaction mixture					
	Undialysed extract			Dialysed extract		
Time in min. ...	0	30	60	0	30	60
2. Phosphatase + vitamin C	5·0	4·2	3·8	5·0	4·1	3·8
3. Phosphatase + vitamin C + Cu	5·0	2·6	1·6	5·0	2·5	1·4

The results show that, unlike kidney and liver phosphatases, the alkaline phosphatase of brain is inhibited by vitamin C-Cu complex irrespective of the purity of the enzyme. This is probably due to the absence of protective substances from the aqueous extracts of acetone-treated brain powder.

The results of the experiments on the protective action of brain extracts against the oxidation of the vitamin are presented in Table VIII.

Table VIII. *Influence of brain extracts on the oxidation of vitamin C*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*p*H 8·9); 5 ml. 0·100% vitamin C; Cu as $CuSO_4, 5H_2O$ (0·062 mg.); total volume, 40 ml.

Oxidation of vitamin C

	mg. of vitamin C in the total volume of reaction mixture							
	Undialysed extract				Dialysed extract			
Time in min. ...	0	15	30	90	0	15	30	60
1. Vitamin C	5·0	4·8	4·6	3·8	5·0	4·6	4·3	4·0
2. Vitamin C + brain extract	5·0	4·8	4·8	4·4	5·0	4·6	4·3	4·0
3. Vitamin C + Cu + brain extract	5·0	3·2	2·6	0·3	5·0	2·8	2·2	1·1
4. Vitamin C + Cu	5·0	3·2	2·6	0·5	5·0	3·0	2·6	1·5

The results show that the protective action of the undialysed extracts of brain is very little compared with those of kidney and liver. The phosphatase is therefore inhibited by the vitamin C-Cu complex, because the vitamin is oxidized by copper under such conditions. Further, just as in the case of dialysed extracts of kidney and liver, brain extracts also accelerate the catalytic oxidations of vitamin C by copper. Experiments on the influence of the vitamin C-Cu complex on the activity of brain acid phosphatase have shown that, like the alkaline phosphatase, the acid phosphatase also is inhibited by the complex.

The influence of glutathione on the vitamin C-Cu inhibition of the activities of the alkaline and acid phosphatases of brain

The results of experiments on the influence of glutathione on the vitamin C-Cu inhibition of the activities of the alkaline and acid phosphatases of brain are presented in Table IX.

Table IX. *Effect of glutathione on the vitamin C-Cu inhibition of brain phosphatase*

Reaction mixture. 10 ml. glycine buffer *M*/5 (*p*H 8·9); 10 ml. 2% Na-β-glycerophosphate; 4 ml. 0·1 *M* $MgCl_2, 6H_2O$; Cu as $CuSO_4, 5H_2O$ (0·062 mg.); vitamin C, 5 mg.; glutathione, 5 mg.; total volume, 41 ml.

	Activity in mg. P in 10 ml. reaction mixture after 4 hr. hydrolysis	
	Alkaline phosphate	Acid phosphatase
1. Phosphatase	0·148	0·284
2. Phosphatase + vitamin C + Cu	0·121	0·172
3. Phosphatase + vitamin C + Cu + glutathione (GSH)	0·121	0·284
4. Phosphatase + glutathione (GSH)	0·138	0·284

Oxidation of vitamin C

	mg. vitamin C in the total volume of reaction mixture							
	Alkaline phosphatase				Acid phosphatase			
Time in min. ...	0	15	30	60	0	30	60	120
2. Phosphatase + vitamin C + Cu	5·0	3·2	2·3	1·0	5·0	3·4	2·7	1·8
3. Phosphatase + vitamin C + Cu + glutathione (GSH)	5·0	4·4	4·2	3·3	5·0	4·8	4·7	4·7

The results show that glutathione (GSH) entirely prevents both the oxidation of vitamin C at *p*H 5·0 and the inhibition of the activity of the acid phosphatase by the vitamin C-Cu complex. The inhibition of the alkaline phosphatase activity is, however, not influenced by glutathione.

The influence of cysteine, cystine, cyanide and sodium hydrosulphite on the inhibiting action of vitamin C-Cu complex

It is well known that the catalytic influence of Cu on the oxidation of vitamin C is entirely or partly removed by glutathione (GSH), cysteine, cystine and cyanide [Hopkins & Morgan, 1936; De Caro & Giani, 1934]. Glutathione, which prevents the oxidation of the vitamin by metal catalysts by virtue of its ability to form a complex with Cu, is found to annul the inhibiting action of the vitamin C-Cu complex on the activity of the phosphatase. It was thought that the inhibition of the activity of the phosphatase by vitamin C might also be annulled by other substances which prevent the oxidation of the vitamin in the presence of Cu. It was found that such compounds as cysteine, cystine and cyanide, when added to the reaction mixture, either diminished or entirely eliminated the inhibiting action of the vitamin C-Cu complex. Sodium hydrosulphite, which is a reducing agent, was also found to protect the phosphatase from the inhibiting action of the complex.

The results are presented in Table X.

Table X. *Influence of cysteine, cystine, cyanide and sodium hydrosulphite on the inhibiting action of vitamin C-Cu complex*

Reaction mixture. 20 ml. acetate buffer *M*/5 (*p*H 5·0); 10 ml. 2% Na-β-glycerophosphate; 10 ml. enzyme (purified by isoelectric precipitation of the impurities at *p*H 4·8); vitamin C, 5 mg.; Cu as $CuSO_4, 5H_2O$ (0·062 mg.); total volume, 51 ml. Cysteine, 5 mg.; cystine, 5 mg.; NaCN, 0·001 *M*; $Na_2S_2O_4$, 0·004 *M*.

Preparation	Substance tested	Reaction mixture	Activity in mg. P in 10 ml. of reaction mixture after 4 hr. hydrolysis
Brain No. 2	Cysteine	Phosphatase	0·231
		Phosphatase + vitamin C + Cu	0·122
		Phosphatase + vitamin C + Cu + cysteine	0·228
		Phosphatase + cysteine	0·220
Brain No. 3	Sodium cyanide	Phosphatase	0·308
		Phosphatase + vitamin C + Cu	0·160
		Phosphatase + vitamin C + Cu + NaCN	0·308
		Phosphatase + NaCN	0·297
Brain No. 4	Cystine	Phosphatase	0·320
		Phosphatase + vitamin C + Cu	0·173
		Phosphatase + vitamin C + Cu + cystine	0·280
		Phosphatase + cystine	0·320
Brain No. 4	$Na_2S_2O_4$	Phosphatase	0·320
		Phosphatase + vitamin C + Cu	0·173
		Phosphatase + vitamin C + Cu + $Na_2S_2O_4$	0·292
		Phosphatase + $Na_2S_2O_4$	0·310

Discussion

The results obtained in the present investigation on the interaction of vitamin C and tissue phosphatases (kidney, liver and brain) have confirmed the view-point developed by the author previously [Giri, 1938, 1 and 2], that the activity of the phosphatase is related to the oxidation of the vitamin. The phosphatases of all tissues are inhibited by the vitamin C-Cu complex, while the vitamin or copper alone has no effect on the activity. Further, it was found that whereas the phosphatase present in purified extracts of tissues is inhibited by the vitamin C-Cu complex, this is not true of the phosphatases present in the crude aqueous extracts under similar experimental conditions. These results are of particular interest since they suggest that the influence of vitamin C on the activity of tissue phosphatases is associated with certain factors present in the tissues, which protect the vitamin against oxidation.

These experiments have an interesting physiological significance. It seems likely that normal tissues always contain sufficient protecting substances to enable the enzyme to act at its full capacity within the cell in the presence of vitamin C and Cu, and that when these substances are removed or their effective concentration decreased by some means, the activity of the enzyme is adversely affected by the vitamin in association with Cu. Thus the interaction of vitamin C, Cu and protective substances like glutathione, which occur together in all tissues, may play an important role in the regulation of the activity of tissue phosphatases.

Summary

1. The activities of the alkaline and acid phosphatases of kidney, liver and brain are inhibited by the vitamin C-Cu complex. Vitamin C or Cu alone has no effect on their activity.

2. The phosphatase activity of crude aqueous extracts of tissues, with the exception of that of brain, is not influenced by the vitamin C-Cu complex, while the activity of extracts purified by dialysis is inhibited by the complex. This difference in the behaviour of the extracts was found to be due to the presence of factors in crude extracts which protect the vitamin against oxidation.

3. The inhibition by vitamin C-Cu complex was entirely or partly annulled by glutathione, cysteine, cystine and reducing agents like NaCN and $Na_2S_2O_4$.

4. It is suggested that vitamin C, Cu and protective substances like glutathione, which occur together in all tissues, play an important role in the regulation of the activity of tissue phosphatases.

I wish to express my thanks to the Indian Research Fund Association and to Dr W. R. Aykroyd, Director, Nutrition Research Laboratories, Coonoor, for providing me with facilities for work in the Laboratories.

REFERENCES

De Caro & Giani (1934). *Hoppe-Seyl. Z.* **228**, 13.
Giri (1938, 1). *Nature, Lond.*, **141**, 119.
—— (1938, 2). *Hoppe-Seyl. Z.* **254**, 126.
Giri & Datta (1936). *Biochem. J.* **30**, 1009.
Hopkins & Morgan (1936). *Biochem. J.* **30**, 1446.

XXXIX. THE CONVERSION OF CAROTENE TO VITAMIN A_2 BY SOME FRESH-WATER FISHES

By R. A. MORTON and R. H. CREED

From the University of Liverpool

(*Received 16 January 1939*)

Freshwater fish contain a substance akin to, but not identical with, vitamin A, which on account of its distribution, chemical, physical and biological properties may properly be designated vitamin A_2. It is differentiated from vitamin A by means of its ultraviolet absorption spectrum and by the antimony trichloride colour test:

	Vitamin A	Vitamin A_2
U.V.	λ_{max} 328 mμ	350 mμ (284 mμ?)
Colour test	λ_{max} 617 mμ [583 mμ] inflexion	693 mμ [645 mμ] inflexion

The available evidence suggests strongly that some of the functions of vitamin A in salt-water fish are fulfilled in freshwater fish by vitamin A_2. The only circumstances under which vitamin A_2 has been found to occur in mammals or birds are those which indicate the presence of vitamin A_2 in the diet (e.g. when freshwater fish are eaten freely) and there is no evidence of the natural synthesis of vitamin A_2 from provitamins except in fishes.

It is accepted that for the mammal α- and β-carotenes (and a few related substances) act as provitamins A, since the administration of such materials results in the relief of symptoms of vitamin A deficiency. If supplied in relative abundance they markedly increase the vitamin A content of the liver.

The origin of the large amounts of vitamin A found in some fishes has never been determined. It is true that phytoplankton contains carotene, but the transformation of carotene into vitamin A has not been directly demonstrated for fishes, nor is there satisfactory evidence that the amount of carotenoid ingested is sufficient to account for the stored vitamin. The constitution of vitamin A_2 remains somewhat speculative and little is known concerning possible or actual precursor substances.

The object of the present series of experiments is to ascertain something of the effect of carotene added to the food of freshwater fish. The literature affords little guidance in the planning of such work. Search was made for a readily accessible freshwater species having the following characteristics:

(1) the fish should be small enough to allow several to live in an aquarium;

(2) the normal vitamin reserves should not be very high;

(3) both vitamin A and vitamin A_2 should be present in easily detectable amounts in the healthy fish on a normal diet.

The most suitable species for a first experiment seemed to be the perch (*Perca fluviatilis*). A number of live fish were secured from Lake Windermere[1] and transported to Liverpool by road in suitable fish tanks. They were transferred on arrival to large glass tanks shaded from direct light and aerated by

[1] The co-operation of the Staff of the Freshwater Biological Association at Wray Castle is gratefully acknowledged.

circulating tap water carrying with it air bubbles. The inlet water was maintained for a time at a slightly higher temperature than that of the room, but after the fish had become habituated to their new surroundings the precaution was found to be unnecessary. It was realized almost at once that too many fish had been placed in the aquaria and a number were removed with a net and pithed. These were then dissected and the different organs tested for carotenoids, vitamin A and vitamin A_2. A few fish died in the first few days and all refused food. This experience is unfortunately common with fish unused to captivity. Search was made for a suitable live food and it was found that the water shrimp *Gammarus neglectus*, which was fortunately available in quantity (River Weaver, Cheshire) proved tempting. After fasting for nearly a fortnight all the perch took the shrimp with avidity and a plentiful supply for about 14 days sufficed to restore a normal appetite.

There were, however, two objections to continuing to feed the fish on water shrimps. In the first place, much work is needed to obtain a sufficient, regular supply of live shrimps and the task of breeding them in the laboratory threatened to be beyond us. Secondly, it was not possible to regard the diet as satisfactory for the experiments which had been planned.

Thus, 28 g. of dead shrimps were completely decomposed by means of alcoholic potash and the non-saponifiable fraction was extracted. It contained a carotenoid pigment (λ_{max} 484, 453, 428 $\sim$ 407 mμ, $\sim$ denoting inflexion) and gave maxima at 617 mμ and 583 mμ with the $SbCl_3$ reagent.

$$E^{1\%}_{1\,cm.}\quad 620\ m\mu\quad 0{\cdot}037$$
$$\phantom{E^{1\%}_{1\,cm.}}\quad 583\ ,,\quad 0{\cdot}029$$

calculated on the weight of shrimp

Similar tests on a further 23 g. of shrimp gave:

$$E^{1\%}_{1\,cm.}\quad 620\ m\mu\quad 0{\cdot}048$$
$$\phantom{E^{1\%}_{1\,cm.}}\quad 583\ ,,\quad 0{\cdot}036$$

and direct absorption spectra confirmed the presence of carotenoid with additional maxima at 344, 316 $\sim$286 and $\sim$276 mμ.

These results are certainly an indication that the water shrimp contains small quantities of material giving tests very similar to those of vitamin A. It would be necessary to carry out a chromatographic analysis of the non-saponifiable fraction in order to be certain which carotenoids are present and to give rigorous proof that the colour test was due to vitamin A rather than to carotenoids. In any case, the shrimp was a poor carrier for adding carotene to the diet. Moreover, it probably contains astacene which may be a vitamin A precursor in some fish.

Various foods were then tried. The digestive glands of *Mytilus* proved on the whole unsatisfactory, minced lean beef was only sparingly eaten, but after some initial reluctance all the fish took blow-fly larvae with great readiness.

At the end of a preliminary period of six weeks the perch were accustomed to the aquaria and could be fed without difficulty. The diet consisted of larvae smeared occasionally with herring-body oil (of negligible vitamin A content). On some days finely chopped raw lean beef was provided.

The next problem concerned the administration of carotene. The provision of an exact amount of carotene in the daily diet of the perch seemed impossible. The object of the experiment was to test the effect of a diet substantially enriched with carotene, and it was necessary to become reconciled to a considerable wastage of carotene in order to be sure that a fair quantity would actually be ingested.

The material used was a sample of leaf carotene containing both α- and β-carotenes but little or no "xanthophyll".

Finely ground solid carotene was placed in a glass dish and clean larvae were picked out with a pair of forceps and dropped into the dish. By gently rubbing the live larvae in the powdered carotene they were easily coated with a minute staining layer which adhered fairly strongly. The coated larvae were fed to the perch two or three at a time and were taken at once. Feeding was on each occasion discontinued as soon as the fish lost interest. It was at first thought that one meal every 48 hours would be best but experience showed that the fish were ready to eat once each day. A little extra food provided at noon on Saturdays had been eaten before Monday morning. Care was taken to remove food debris from the aquaria and to ensure constant aeration. A group of fish maintained in a separate tank received a diet as nearly as possible identical with that supplied to the experimental group, except that the controls received no added carotene.

After a period of two months the controls were removed by means of a net and at once killed by pithing. They were then dissected and the various portions assembled for analysis. Each sample was refluxed with alcoholic potash and the non-saponifiable fractions were extracted with ether. The ether extracts were dehydrated by treatment with pure alcohol and nitrogen on a water bath. The non-saponifiable fractions were then subjected to the $SbCl_3$ colour test and the vitamin concentrations estimated by spectroscopic examination.

Experimental data

Perch from Lake Windermere—not fed in captivity

(*a*)	(*b*)
8 fish: 4♂, 4♀	3 fish

Average wt. 38 g., average length 14·9 cm.

Livers: 3·274 g., average 0·42 g.

$E^{1\%}_{1\text{ cm.}}$ { 693 mμ 0·04; 640 ,, 0·075; Feeble maxima at 595 and 560 mμ

Viscera (comprising hearts, spleens, gonads, mesenteries): 20·3 g., average 2·2 g.

$E^{1\%}_{1\text{ cm.}}$ { 695 mμ 0·01; 636 ,, 0·011; 556 ,, 0·09

Alimentary tracts: 6·98 g., average 0·87 g.

$E^{1\%}_{1\text{ cm.}}$ { 693 mμ 0·008; 600 ,, Transient; 620 ,,

Eyes and bodies both gave feeble colour tests

1·253 g., average 0·42 g.

$E^{1\%}_{1\text{ cm.}}$ { 693 mμ 0·018; 640 ,, 0·044; 605 ,, 0·053; 560 ,, 0·043

Eyes	Feeble blue colour
Pyloric caeca	,,
Intestines	,,
Mesenteric fat	,,
Gonads	No blue colour
Hearts	,,
Spleens	,,
Stomachs	,,

Controls: 6 fish fed in captivity, no added carotene

Livers: 3·71 g., average 0·62 g.

$E^{1\%}_{1\text{ cm.}}$ { 693 mμ 0·064; 620 ,, 0·040

Mesenteries: 3·58 g., average 0·6 g.

$E^{1\%}_{1\text{ cm.}}$ { 695 mμ 0·0116; 655 ,, 0·0124; 620 ,, 0·0124; 580 ,, 0·0085

Eyes	Very faint blue colour
Pyloric caeca	No blue colour
Hearts	,,
Spleens	,,
Stomachs	,,
Intestines	,,
Gonads	,,

Perch on a diet enriched with carotene

3♀ immature, 3♀ adult, 2♂ adult
Average wt. 36·3 g., average length 15·8 cm.
Livers: 4·28 g., average 0·61

$E^{1\%}_{1\,cm.}$ { 693 mμ 0·206 ; 620 „ 0·086 only 693 mμ band seen

Mesenteries: 4·74 g., average 0·68 g.

$E^{1\%}_{1\,cm.}$ { 645 mμ 0·0086 ; 605 „ 0·0096 ; 592 „ 0·0086

Pyloric caeca: 2·83 g.	Faint blue colour
Intestines: 3·86 g.	Faint blue (greenish blue)
Gonads: 17·43 g.	Faint blue
Eyes: 5·78 g.	„
Stomachs: 2·32 g.	No blue colour
Hearts: 0·47 g.	„
Spleens: 0·28 g.	„

It is clear that vitamin A and vitamin A_2 occur preferentially in the livers. Although in an absolute sense the concentrations are low, the amount of vitamin A_2 is very definitely increased as a result of the administration of dietary carotene.

	$E^{1\%}_{1\,cm.}$ 693 mμ
Windermere fish feeding naturally	0·04
	0·018
Controls	0·064
Carotene-fed perch	0·206

The control fish had, as it turned out, rather larger reserves than those fresh from the open lake. There can be little doubt that the threefold increase in vitamin A_2 and the twofold increase in vitamin A are consequent upon the administration of carotene.

A few months later, perch from a different locality became available after feeding naturally until July, 1938. They had thus had the benefit of the photosynthetic activity in spring and early summer and it would be reasonable to expect that they had obtained full access to the natural provitamins A.

Perch from Aberdeen

20 fish, average wt. 225 g.

Livers 73 g. (average 3·65 g.). After refluxing with alcoholic potash and extraction with ether 0·33 g. of non-saponifiable matter was isolated:

		On non-sap.	On liver	
$E^{1\%}_{1\,cm.}$	693 mμ	132	0·6	Colour test
	617 „	46	0·21	
	351 „	36·4	0·165	u.v. absorption

It will be seen that the ratio $E^{1\%}_{1\,cm.}$ 693/617 mμ is 2·87, showing that vitamin A_2 is present in much larger amount than vitamin A. The absolute quantity, *c.* 120 p.p.m., is not high but is nevertheless three times as great as that shown by the carotene-fed perch and some nine times as great as that shown by the controls. The ultraviolet absorption spectrum shows, in addition to the 351 mμ maximum characteristic of vitamin A_2, a second band at 284 mμ which may be due to a third absorbing entity in the non-saponifiable fraction.

It will be noticed that these perch were on the average much larger (six to seven times by weight) than those obtained from Windermere. The livers from the

present batch of perch averaged 3·65 g. as against 0·6 g. for the experimental fish. The much higher storage reserves of vitamin A_2 in the larger and (probably) older fish is in good accord with experience obtained with the vitamin A contents of cod and halibut livers. It seems likely that in any year the fish tends to show a positive balance in its vitamin A or A_2 economy resulting in greater storage with age.

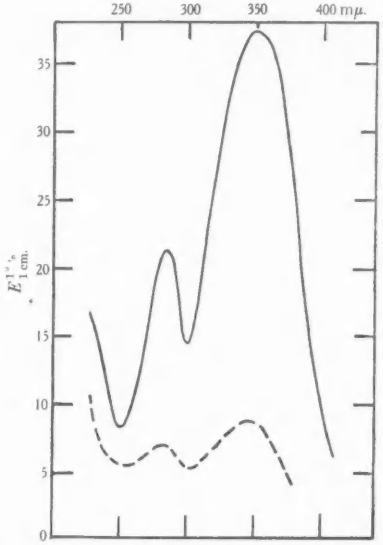


Fig. 1. Ultraviolet absorption spectrum of non-saponifiable fractions from (*a*) perch liver oil ——; (*b*) perch intestinal oil – – –.

Stomachs. 30 g. of tissue yielded 0·1318 g. (4393 p.p.m.) of non-saponifiable matter. A 13·2 % solution of the "non-sap." diluted with 10 vol. of the $SbCl_3$ reagent gave a blue solution by means of which it was just possible to recognize the 693 mμ maximum but not the 617 mμ band. The vitamin content of the stomachs must therefore have been negligibly small.

Eyes. The whole eyes from 20 perch weighed 47 g. Prolonged treatment with hot alcoholic potash followed by the usual extraction process gave 0·9563 g. (20,350 p.p.m.) of non-saponifiable matter. This material gave a good blue colour with the $SbCl_3$ reagent:

$$E^{1\%}_{1\,cm.}\begin{cases}693\ m\mu & \text{Not measurable}\\ 642\ ,, & 0{\cdot}315\\ 600\ ,, & 0{\cdot}294\\ 565\ ,, & 0{\cdot}28\end{cases}$$

Three absorption bands were seen clearly. The eyes evidently contain small quantities of both vitamin A and vitamin A_2 although the rest of the non-saponifiable fraction exerts marked inhibition in the colour test. The eyes probably yielded 2 p.p.m. vitamin A and 3–4 p.p.m. vitamin A_2.

Intestines. 60 g. yielded 0·353 (5900 p.p.m.) of non-saponifiable matter:

$$E^{1\%}_{1\,cm.}\begin{cases}\text{On non-sap.}\\ 693\ m\mu \quad 23\\ 617\ ,, \quad 9{\cdot}3\end{cases}$$

The ratio $E^{1\%}_{1\,cm.}$ 693 mμ/617 mμ is 2·47, i.e. vitamin A_2 does not preponderate quite so much as in the livers. The ultraviolet absorption shows two maxima, one near 350 mμ and the other near 285 mμ. The relative intensities of these two maxima in the intestinal non-saponifiable fraction are so strikingly different from the relative intensities shown by the liver "non-sap." that little doubt can remain that the two maxima belong to distinct compounds.

Mesenteric fat. A good deal of mesentery could be detached from the ileum of each fish. 68 g. yielded 0·098 g. of "non-sap.":

		On non-sap.
$E^{1\%}_{1\,cm.}$	693 mμ	22·5
	617 „	10·7

corresponding to *c.* 2·5 p.p.m. vitamin A and *c.* 6·5 p.p.m. vitamin A_2.

The fact that carotene can act as provitamin A and provitamin A_2 for the perch seems established from this work, but the failure to raise the storage levels to that observed in larger fish feeding naturally is at first sight disturbing. There is little reason to suppose that carotene is the sole precursor substance. In fact the amounts of carotene available in the natural diet are rather surprisingly small.

Thus a sample of 53·8 g. of zooplankton supplied from Windermere (by courtesy of the Freshwater Biological Research Station, Wray Castle) after saponification yielded a fraction unmistakeably containing carotene but so contaminated with other absorbing substances as to preclude a quantitative assay. The acids recovered from the soaps were deeply coloured and gave numerous absorption bands (λ_{max} 271, 283, 301, 317, 330, 349, 377, 405, 450, 482 mμ) indicating the presence of conjugated polyenes. The quantities available did not permit of fractionation.

A further experiment was made on dace fed on blow-fly larvae with and without added carotene:

Experiments on dace

Controls		Diet enriched with carotene	
5 fish		4 fish	
Livers: 8·78 g.		7·4 g.	
$E^{1\%}_{1\,cm.}$ 693 mμ	0·19	$E^{1\%}_{1\,cm.}$ 693 mμ	0·73 Colour
620 „	0·19	620 „	0·65 test
		345 „	0·28 u.v. test
Vitamin A	*c.* 38 p.p.m.	Vitamin A	*c.* 130 p.p.m.
Vitamin A_2	*c.* 38 p.p.m.	Vitamin A_2	*c.* 146 p.p.m.
Stomachs: 1·45 g.		1·5 g.	
$E^{1\%}_{1\,cm.}$ 693 mμ	0·011	$E^{1\%}_{1\,cm.}$ 693 mμ	0·35
620 „	0·027	620 „	0·70
		583 „	0·38
Vitamin A	5·4 p.p.m.	Vitamin A	140 p.p.m.
Vitamin A_2	2·2 p.p.m.	Vitamin A_2	70 p.p.m.
Intestines: 1·25 g.		1·14 g.	
$E^{1\%}_{1\,cm.}$ 620 mμ	0·027	$E^{1\%}_{1\,cm.}$ 693 mμ	0·07
		620 „	0·225
Vitamin A	5·4 p.p.m.	Vitamin A	45 p.p.m.
		Vitamin A_2	14 p.p.m.
Rest of viscera: 8·1 g.		5·3 g.	
Vitamin A	2·4 p.p.m.	Vitamin A	4 p.p.m.
Vitamin A_2	2·0 p.p.m.	Vitamin A_2	3·2 p.p.m.
Gonads: 27·4 g.		40·8 g.	
Vitamin A	0·84 p.p.m.	Vitamin A	1 p.p.m.
Vitamin A_2	0·74 p.p.m.	Vitamin A_2	1 p.p.m.
Eyes: Trace of vitamins A and A_2		Trace of vitamins A and A_2	

The work on dace shows a substantial increase in the amounts of both vitamins A and A_2 as a result of adding carotene to the diet. The marked increase in the alimentary tract is striking and suggests that the possibility of conversion of carotene in the stomach and intestines requires further investigation.

An attempt was made to extend the experiments to chub. The controls (2) on the carotene-free diet were unsatisfactory, as they could not be induced to eat freely. They may have been injured before arrival, but when it was seen that they were not doing well they were removed by means of a net, pithed and examined. Their vitamin reserves were low. The fish receiving a diet enriched with carotene compared badly with later specimens feeding naturally:

	Chub fed on blow-fly larvae plus carotene			Freshly caught chub (Mar. 1938)		
		P.p.m. vitamin			P.p.m. vitamin	
	Wt. g.	A	A_2	Wt. g.	A	A_2
Livers	8·4	39	33	6·9	120	50
Intestines	3	4·3	1·5	1·33	75	43
Stomachs	4·2	3·2	1·6	1·31	110	6
Rest of viscera	4·1	0·2	1·9	4·73	94	42
Eyes		Traces			Traces	
Gonads		Traces			Traces	

The chub did not take kindly to captivity, and this experiment is unsatisfactory. The striking difference between the contents of the alimentary tracts in respect of vitamins A and A_2 is worth emphasis.

Summary

Perch and dace in captivity appear to thrive for a considerable period on a diet of blow-fly larvae. When this diet is enriched with carotene for a few weeks the store of vitamins A and A_2 increases considerably. From this it is concluded that carotene acts as provitamin for the formation *in vivo* of both vitamins. Whatever the constitution of vitamin A_2 may be it cannot be very different from that of vitamin A.

We are indebted to Dr J. A. Lovern, Dr F. J. Daniel, Mr G. W. Simpkins and Mr F. Dawson for valuable assistance. Thanks are also due to the Medical Research Council for a grant which enabled this work to be carried out.

XL. THE DISTRIBUTION OF VITAMINS A AND A_2. II

By JOHN ARNOLD LOVERN, RICHARD ALAN MORTON and JAMES IRELAND

From the Torry Research Station, Aberdeen, and the Chemistry Department, University of Liverpool

(*Received 25 January 1939*)

In the present phase of research on the A-vitamins two facts merit special attention. One concerns the distribution of the vitamins in respect of the absolute amounts found at different sites in different species and the other the relative proportions of the two vitamins.

The first fact is that a great deal of vitamin occurs in the absorptive parts of the alimentary tracts of many, but not all, species of fish. The second is that vitamin A_2, which is practically confined to fishes, shows in all species a distribution over the body which differs appreciably from that of vitamin A. Vitamin A_2 predominates over vitamin A only in fresh water fishes; it has, however, been detected in birds subsisting on fish from inland waters.

Earlier work [Edisbury *et al.* 1938] on the distribution of the two vitamins has been extended.

Experimental

Sturgeon (Acipenser sturio)

A large sturgeon 10 ft. 6 in. long was landed at Aberdeen in May, 1938. The viscera were obtained and extracted in the laboratory.

The pyloric caeca, which form a compact mass in the sturgeon, were missing and had probably been thrown away by the fish merchant. The stomach was not freed from adhering mesentery but showed a high oil content which could not be attributed to mesenteric fat.

Table I

	Wt. g.	Oil %	Wt. of oil g.	Vitamin in oil %	Wt. vitamin g.	Vitamin A p.p.m. in tissue
Liver	3941	37·2	1466	1	14·66	*c.* 3000
Intestines*	1541	1·9	29·3	10	2·93	1900
Stomach	233	25·7	60	0·01	0·006	26
Spleen	145	4·8	6·96	0·015	0·001	7
Mesentery	75	24·0	18	0·006	0·0011	14

* Minus pyloric caeca.

Table II. *Spectroscopic tests on oils*

		Liver oil	Intestinal oil	Spleen oil	Stomach oil	Mesenteric oil
$E^{1\%}_{1\,cm.}$ 693 mμ	Colour test	11	40	Weak	Not detectable	Not detectable
$E^{1\%}_{1\,cm.}$ 617 ,,	Colour test	47	509	0·36	0·18 (600 mμ)	0·12 (600 mμ)
$E^{1\%}_{1\,cm.}$ 583 ,,	$SbCl_3$	26	272	0·38	0·23 (572 mμ)	0·16 (572 mμ)
$E^{1\%}_{1\,cm.}$ 325 ,,	Direct absorption	17	157	—	—	—
		No inhibition of colour test		Marked inhibition of colour test with $SbCl_3$		
Ratio $E^{1\%}_{1\,cm.}$ 617/693 mμ		4·3	12·7			

This large sturgeon contained some 30 g. of vitamin A esters and perhaps 7 g. of vitamin A_2 esters in its liver. The complete intestines must have contained at least 6 g. of vitamin A esters and perhaps 0·5 g. of vitamin A_2 esters. These quantities are large for a single fish and support the possibility previously suggested that the A-vitamins possess functions not hitherto recognized.

Possibly vitamin A_2 is passed on to the liver from the intestines more readily than vitamin A, since the ratio A/A_2 is much higher in the intestines than in the liver. This is also true of salmon, trout and halibut [Edisbury *et al.* 1938]. The distribution of the two vitamins suggests that they may not exert identical functions at all sites.

Lampern (Petromyzon fluviatilis)

In order to illustrate the kind of contrast which has already been noticed between, for example, eels [Edisbury *et al.* 1937] and halibut [Edisbury *et al.* 1938], the sturgeon may be compared with the freshwater lamprey. Three sets of data were obtained and gave results in fair agreement.

Lamperns (Worcester), Nov. 1937. 6 ♂, 6 ♀. Total wt. 439 g. Average wt. 36·6 g.

		Digestive glands	Fore-gut	Hind-gut	Gonads and excretory glands	Eyes	Bodies: Head and thorax	Bodies: Abdomen and tail
Wt. g. ...		6·45	1·4	1·28	35·4	1·0	109·5	284·5
$E^{1\%}_{1\,cm.}$	693 mμ	Not seen	Not seen	0·16	0·13	—	0·01	0·007
	617 ,,	0·185	0·37	1·7	1·18	0·015	0·03	0·016
	583 ,,	0·12	0·2	0·9	0·6	—	0·023	0·012
	325 ,,	—	—	—	—	—	0·027	0·016
Approx. wt. of vitamin A, mg.		0·24	0·1	0·4	8·26	0·003	0·04	0·08
Vitamin A, p.p.m.		37	70	312	233	3	0·37	0·38

It is noteworthy that vitamin A predominates over vitamin A_2 and is very widely distributed in small amounts.

Dogfish (Squalus acanthias)

Some species are noteworthy for possessing abnormally low vitamin A reserves in the liver. Our experience of dogfish liver oil is that it commonly contains very little vitamin but it would be rash to generalize because we have examined one specimen (from Newfoundland) which was unusually rich. If a species tends to store little vitamin A in the liver, the present work makes it interesting to examine its intestines. We therefore retained a quantity of such material from dogfish.

558 g. of dogfish intestines were decomposed by means of alcoholic potash; 2·25 g. of non-saponifiable extract, obviously mainly sterol, were obtained. This material gave the following tests:

$$E^{1\%}_{1\,cm.}\begin{cases}693\ m\mu & 0{\cdot}46\\ 617\ ,, & 8{\cdot}5\\ 580\ ,, & 5{\cdot}27\end{cases}$$

The vitamin A content of the "non-sap." is of the order 0·2 % so that the intestines contain:

c. 4000 p.p.m. of sterol,
8 p.p.m. of vitamin A,
0·3 p.p.m. of vitamin A_2.

The contrast with halibut intestines containing up to 35,000 p.p.m. vitamin A is very striking and suggests a radical difference between the two species in respect of the mechanism of fat absorption (see Part III in this *Journal*, p. 330).

We have already noted [Edisbury *et al.* 1938] a marked decrease in the vitamin A content of the intestines of halibut as the absorptive function decreases in importance towards the vent. This has been confirmed. Thus the entire rectal caecum of one fish yielded 0·8 % of oil containing 4·25 % of vitamin, corresponding to 340 p.p.m., as against 33,000 p.p.m. for the absorptive parts of the gut.

The stomach of halibut is likewise poor in oil. The entire stomach of one fish yielded 0·5 % of oil, 26·7 % of which was non-saponifiable matter, mainly cholesterol. The vitamin A content was of the order 2 p.p.m. The stomach "oil", if dissolved in a suitable solvent to saturation at the boiling point, yields a well crystallized mixture of sterol and sterol esters on cooling. A halibut caught in Sept. 1938 was obtained with viscera in a good state of preservation. The stomach tissue was split up into various coats (see Part III in this *Journal*, p. 330) and the amount of non-saponifiable matter was determined:

	Wt. g.	Non-sap. p.p.m.
Layer 1 (inside)	214	3900
2	596	3200
3	263	4150
4	562	3070

The proportion of sterol did not exceed that usual in muscle cells, and the lining of the stomach contained little or no fat and practically no vitamin A.

The sterols from the various layers were recrystallized and some were obtained pure enough to warrant quantitative determinations of provitamin D content (probably 7-dehydrocholesterol). Cholesterol from cod liver oil, salmon liver oil and similar sources shows the ergosterol-7-dehydrocholesterol absorption bands at 293·5, 281·5 and 270 mμ, $E^{1\%}_{1\,\mathrm{cm.}}$ 281·5 mμ 0·1 to 0·3 corresponding to some 0·03–0·1 % of provitamin. The sterol from halibut stomach also shows these bands but at much higher intensity, $E^{1\%}_{1\,\mathrm{cm.}}$ 281·5 mμ 6·2 and 4·5. No explanation of this has been found, but it is possible that the provitamin D is a product of dehydrogenation of cholesterol occurring in the tissue.

Halibut spleen from the same fish was also studied. It yielded *c.* 5300 p.p.m. of non-saponifiable matter, *c.* 380 p.p.m. of vitamin A and *c.* 35 p.p.m. of vitamin A_2. Recrystallization of the non-saponifiable matter from methyl alcohol gave sterol in good yield. One further recrystallization gave a snow-white sample of cholesterol and an almost colourless mother liquor. Spectroscopic examination of both sterol and mother liquor revealed highly selective absorption of an unexpected type. The broad persistent absorption band with λ_{max} 275 mμ showed no sign of resolution into the narrow bands characteristic of ergosterol and dehydrocholesterol. The intensity of absorption, $E^{1\%}_{1\,\mathrm{cm.}}$ 275 mμ 4·6, in the recrystallized sterol was by no means negligible and perhaps indicates that the cholesterol was contaminated with 1–2 % of a substance not so far identifiable.

Similar experiments on bullock spleen and sheep spleen failed to show the presence of this absorbing impurity in mammalian spleen.

Bullock spleen:

Non-sap. 5200 p.p.m.
Carotene *c.* 1·5 p.p.m.

Recrystallized sterol showed three bands corresponding with those of ergosterol and 7-dehydrocholesterol (0·02 % in the cholesterol).

Sheep spleen:

Non-sap. 4800 p.p.m.
Carotene 0·26 p.p.m.

Recrystallized sterol contained *c.* 0·06 % of "dehydrocholesterol".

The amount of vitamin A in the bullock and sheep spleens was extremely minute.

Vitamin A in sea-birds

In view of the considerable, though variable, amounts of vitamin A occurring in the absorptive parts of the intestines of fishes, it seemed desirable to study its distribution in birds subsisting mainly on fish. The herbivorous mammal does not retain more than a few parts per million of vitamin A in its intestines. The following data must be regarded as preliminary to further work. The figures indicate, however, the presence of appreciable amounts of vitamin in the intestines.

Herring gull (Larus argentatus)

The liver (29 g.) yielded 0·172 g. of non-saponifiable matter, 23 % of which (0·04 g.) consisted of vitamin A corresponding to 1380 p.p.m. in the tissue. Vitamin A_2 could not be detected.

The intestines (43 g.) yielded 0·194 g. of non-saponifiable matter containing 2·9 % (0·0056 g.) of vitamin A or 130 p.p.m. Vitamin A_2 was again absent.

The heart and lungs both gave extremely low values (< 1 p.p.m.) for vitamin A.

Skua gull (Megalestris catarrhactes)

The livers from 8 birds weighed 300 g. and yielded 2·2 g. of non-saponifiable extract containing 32 % (0·7 g.) of vitamin A. This corresponds to *c.* 2300 p.p.m.

The intestines (345 g.) contained 1·8 g. of non-saponifiable matter, 4 % of which was vitamin A. This is equal to 208 p.p.m. of vitamin.

The hearts, eyes and lungs all contained less than 1 p.p.m. vitamin A. No vitamin A_2 was detected in any part of the birds.

Gannet (Sula bassana)

One bird was dissected. The lungs were very large (74 g.) and gave 0·341 g. of non-saponifiable extract, 19 % (0·065 g.) of which was vitamin A. This corresponds to 870 p.p.m.

The liver (5·5 g.) was abnormally small and only contained a few p.p.m. of vitamin A.

The intestines (54 g.) yielded 0·231 g. of non-saponifiable matter mainly sterol and the vitamin A content did not exceed 10 p.p.m. The heart (30·5 g.) appeared to contain 0·7 p.p.m. vitamin A.

If these results are to be believed, the lungs take precedence over the liver and intestines as sites for vitamin A deposits in this species. The data are very surprising but we have found no valid reason for their rejection. A number of gannets will be studied as they become available.

Summary

A large sturgeon yielded 1·47 kg. of liver oil containing 1 % of vitamin A, whilst part of the intestines gave 29·3 g. of oil containing 10 % of vitamin. The liver contained *c.* 30 g. vitamin A esters and 7 g. vitamin A_2 esters. The intestines probably contained 6 g. and 0·5 g. of vitamin A and vitamin A_2 esters

respectively. The ratio vitamin A/A_2 was much higher in the intestines than in the liver.

Lamperns are not rich in vitamin A but small quantities occur at a number of sites. Vitamin A predominates over vitamin A_2.

The dogfish, which yields a liver oil poor in vitamin A, also possesses extremely little in its intestines. Those parts of the alimentary tract of the halibut which take little part in the assimilation of fat are likewise poor in vitamin and it may be that the dogfish and some other species do not use vitamin A in absorption to the same extent as the halibut, cod and salmon.

A beginning has been made with the study of the distribution of provitamin D in the cholesterols obtained from different organs. The non-saponifiable fraction of spleen is noteworthy for the occurrence of small quantities of a substance with λ_{max} 275 mμ. This substance has not so far been detected in mammalian spleens.

Sea birds (herring gull and skua) have much more vitamin A in their intestines than such animals as the rabbit. The case of the gannet is surprising in that its lungs contain relatively large quantities of vitamin.

The work as a whole leads to the conclusion that vitamins A and A_2 probably do not replace one another with equal readiness in all functions. The enormous variations in the vitamin A content of fishes' intestines make it probable that the mechanism of assimilation is different when vitamin A is a major intestinal constituent from that obtaining when the vitamin is a trace constituent.

The authors wish to express their thanks to the Medical Research Council for a grant towards the expenses of this work and to Mr R. H. Creed for valuable technical assistance.

REFERENCES

Edisbury, Lovern & Morton (1937). *Biochem. J.* **31**, 416.
—— Morton, Simpkins & Lovern (1938). *Biochem. J.* **32**, 118.

XLI. THE DISTRIBUTION OF VITAMINS A AND A_2. III

By JOHN ARNOLD LOVERN and RICHARD ALAN MORTON

From the Torry Research Station, Aberdeen, and the Chemistry Department, University of Liverpool

(*Received 25 January 1939*)

The presence of large quantities of vitamin A in the intestines of certain fish was recently recorded [Lovern *et al.* 1937; Edisbury *et al.* 1938]. Its distribution in various parts of the alimentary tract was studied and, in the case of the halibut, the stomach was found to contain only traces of the vitamin whereas the "fatty" material extracted from the pyloric caeca, duodenum and ileum contained up to 70% of vitamin A esters.

The object of the present paper is to record the distribution of vitamin A and its congener vitamin A_2 in the different coats of the intestinal tube.

The problem has been approached in two ways:

(*a*) Photomicrography of sections cut transversely.

(*b*) Determination by spectroscopic methods of the vitamin content of the different layers of tissue after mechanical separation.

Histological work directed towards ascertaining the distribution of vitamin A demands a departure from normal technique because the solvents which might be used for clearing the sections are capable of dissolving out the vitamin, whilst the usual methods of fat-staining are unlikely to discriminate sharply between glycerides and vitamin A esters. We were therefore led to study the possibilities of ultraviolet photomicrography. Vitamin A absorbs radiations in the near ultraviolet region but transmits freely the longer wave-lengths of visible light. In principle, therefore, localized deposits of vitamin A should be detectable by the comparison of photographs taken by means of ultraviolet light and visible light respectively, using the same section and microscope setting for both experiments.

The ordinary microscope transmits ultraviolet rays in the region 340–400 mμ and although the peak of the vitamin A absorption (328 mμ) falls outside this range, the long-wave side of the broad absorption band includes a sufficient part of the spectrum transmitted.

The special glass used in ultraviolet fluorescence cabinets (Wood's glass or Chance's ultraviolet glass) transmits practically no visible light but is fairly transparent towards near ultraviolet rays. If, after taking a photomicrograph in the usual way with visible light, a sheet of such glass is placed between the light source and the microscope, a second photograph may be taken by means of ultraviolet light. The exposure, of course, will need to be much longer.

A simple test of the method is easily made. If three small drops of oil are placed adjacent to one another on a cover slip as follows:

Drop 1. Olive oil (no vitamin A),
Drop 2. Cod liver oil (low vitamin A content),
Drop 3. Halibut liver oil (high vitamin A content),

and photographed in the ways suggested above it is found that the ordinary negative shows a similar degree of blackening for the three drops, whereas the negative obtained with ultraviolet radiation exhibits a decreasing blackening in the order 1, 2, 3.

Experimental

Preliminary experiments were carried out on the intestines of a large halibut. The fish was dissected at Aberdeen and the intestines were sent to Liverpool, in the frozen state, in a large Dewar flask. The material arrived still frozen and the flask could not be emptied until the contents had been thawed. Sections were cut by hand and mounted in a large vertical enlarging lantern. Photographs taken by projection using screened and unscreened light gave promising results. It was realized, however, that the intestines were not too well preserved and that autolysis had occurred, particularly on the inside. Further, the sections were not clear enough, the magnification was inadequate and the ultraviolet pictures were not in good focus.

In order to cope with these difficulties it was arranged to carry out further work at sea on absolutely fresh material. The only method of clearing the sections which seemed satisfactory was that of MacConaill [1937–8], namely, immersing the freshly cut sections in a homogeneous mixture of glucose and golden syrup. It was found that the ordinary microscope could readily be adapted for use. It was employed horizontally, the mirror being dispensed with. A "pointolite" bulb provided sufficient ultraviolet radiation for the purpose. These preliminary details having been settled, fishing for halibut was undertaken in Shetland waters in May, 1938. Adverse weather conditions unfortunately limited both the catch and the experimental work but enough was done to demonstrate the need for fresh material and for fairly thick sections.

A little later the intestines of a very large halibut became available at Aberdeen in good condition and, as a result of previous experience, much more satisfactory photographs were obtained. The best results were obtained with hand-cut slices, 1–2 mm. thick before clearing, though with such sections it was not possible to obtain a clear picture of the detailed structure. Many pairs of photographs were, however, obtained and in the best of these it was evident that the mucosal coats were much more opaque to ultraviolet light than to visible light, whilst the muscle layers showed little sign of such selective absorption.

The technique is capable of considerable refinement but in the present work it was thought better to approach the problem from a different angle.

Attempts were made, therefore, to isolate the mucosal tissue from the underlying muscle and skin, in order to extract the oils and determine the respective vitamin A potencies.

It was clear from the earliest trials that the inside coats were richer than the outside ones, both in respect of fat and vitamin, but, owing to autolysis, the results lacked quantitative significance. For work of this kind it is desirable to use the very fresh viscera of a large halibut. The thick gut was opened longitudinally and the various coats were separated. Samples of such materials were (*a*) placed in alcohol and sent from Aberdeen to Liverpool for examination, and (*b*) extracted in bulk at Aberdeen. In general, lower values were obtained with the alcohol-treated samples, suggesting some destruction of vitamin.

Terminology. A thorough description of the anatomy and histology of the alimentary tract in the king salmon is given by Greene [1912]. We have already found that salmon, like halibut, contain considerable quantities of vitamin A in the intestines, and, as no comparable work on halibut was available to us, we have adopted the terminology used by Greene and supported by detailed drawings of histological sections.

The different coats in the intestines and pyloric caeca, from the lumen to the outer wall, are described as follows:

I. *Mucosa:* (*a*) Epithelium,
(*b*) *Tunica propria*,
(*c*) *Stratum compactum*,
(*d*) *Stratum granulosum*.

II. *Muscularis:* (*a*) *Circularis*,
(*b*) *Longitudinalis*.

III. *Serosa:* (*a*) *Serosa* proper,
(*b*) *Sub-serosa*.

The epithelial coat exhibits much folding but no villi and consists mainly of columnar epithelial cells, with mucous cells and wandering cells of the leucocyte type.

The *tunica propria* is made up of supporting connective tissue extending from the bases of the epithelial cells to the *stratum compactum*. It consists of white, fibrous tissue of the areolar type.

The *stratum compactum* is described by Greene as consisting of dense, non-fibrous connective tissue. The inner surface has projecting strands of the same type and the outer surface possesses two or three times as many of these processes, which form a network extending to the muscle coat. "Nowhere does there seem to be any direct opening or break through the thicker portion of the *stratum compactum* except where blood vessels penetrate this coat."

The *stratum granulosum* consists of granule cells enmeshed in the processes of the *stratum compactum*.

The muscle coats have been taken together in this work, as have those of the *serosa*, which consist of an outer "skin" and supporting connective tissue.

Two large halibut, one caught in May (A) and the other in September, 1938 (B), provided suitable material. In fishes generally the intestinal divisions are not so clearly differentiated as in mammals, but in this work a definite thinning of the intestinal wall was taken to mark the transition of duodenum to ileum.

After washing out food residues, a quantity of slimy matter, consisting of partly broken down mucosal epithelium, could be removed by very lightly scraping the inside of the gut. The wet weight of samples was taken before the addition of alcohol.

Mucosal epithelium. Sample 1*a* (18·76 g.) from the combined pyloric caeca and duodenum of fish A was covered with absolute alcohol and later ground in an agate mortar with sand and anhydrous sodium sulphate. The whole mass was thoroughly extracted with ether, giving 0·68 g. of fatty material (3·6 %). Spectroscopic examination showed a vitamin A content of about 5·3 %. The vitamin A content of the wet epithelium was therefore 0·19 %, i.e. 1900 p.p.m. The ratio $E^{1\%}_{1\,\mathrm{cm.}}$ 617 mμ/693 mμ, representing the proportion of vitamin A to vitamin A_2, was 10·8.

Sample 1*b*. The whole of the slime collected from the pyloric caeca, duodenum and ileum of fish A was subjected to direct fat extraction. The yield was 2·2 % of an oil containing 12 % of vitamin A, corresponding to 2680 p.p.m. The ratio $E^{1\%}_{1\,\mathrm{cm.}}$ 617 mμ/693 mμ (613/51) was about 12.

Sample 1 *c*. Slime (40·04 g.) from the combined intestines of fish B, after grinding with sand and extraction with alcohol followed by ether, gave 3·01 g. of clear oil (7·5 %). The spectroscopic characteristics of the oil:

$$E^{1\%}_{1\,\mathrm{cm.}}\begin{cases}693\ \mathrm{m}\mu & c.\ 14\\ 617\ ,, & 170\\ 583\ ,, & 92\end{cases}\ \mathrm{SbCl_3\ colour\ test}$$

$$325\ ,,\quad 54\ \text{u.v. absorption}$$

were quite normal and corresponded to about 3·45 % of vitamin A, or 2590 p.p.m. in the wet slime.

Tunica propria. After removal of the epithelial slime, this coat could be removed by harder scraping.

Sample 2*a*. This experiment was designed to secure a sample of oil in a state as near as possible to the natural one. The alcohol-preserved tissue from the pyloric caeca and duodenum of fish A was ground in an agate mortar with sharp sand and extracted in the cold with ether, a little alcohol being added occasionally to replace that lost in the successive extractions. 16·18 g. (wet weight) of tissue yielded 1·4 g. of oil, but the extraction was probably not quantitative. It contained 30 % of vitamin A.

Sample 2*b*. Direct extraction of the main bulk of the *tunica propria* from the pyloric caeca and duodenum yielded about 11 % of oil of similar potency:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 140 \\ 617\ \text{,,} & 1545 \\ 583\ \text{,,} & 825 \\ 325\ \text{,,} & 460;\ 445{\cdot}5 \end{cases}$$

Taking these figures to correspond to some 30 % of vitamin A (*c.* 60 % vitamin A esters) the *tunica propria* contained some 33,000 p.p.m. The vitamin A_2 content is of the order 3000 p.p.m.

Sample 2*c*. The *tunica propria* scrapings (38 g.) from the ileum of fish A were preserved in alcohol and later, after grinding with sand and sodium sulphate, were exhaustively extracted with alcohol and ether, giving 11 % of pale, clear oil. The spectroscopic data:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 93{\cdot}5 \\ 617\ \text{,,} & 1650 \\ 583\ \text{,,} & 900 \\ 325\ \text{,,} & 485 \end{cases}$$

correspond to 32·5 % of vitamin A, or 35,750 p.p.m. in the tissue.

Sample 2*d*. 37·4 g. of the *tunica propria* from the combined intestines of fish B gave 3·85 g. of oil (10·3 %) having the following characteristics:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 56 \\ 615\ \text{,,} & 900 \\ 583\ \text{,,} & 500 \\ 325\ \text{,,} & 275 \end{cases}$$

or 18 % vitamin A, corresponding to 18,500 p.p.m. in the tissue.

Stratum compactum *and* stratum granulosum. After scraping off the *tunica propria* it was found possible to detach the next coat with a sharp knife and to peel it off in long strips. This coat was taken to include both the *stratum compactum* and the enmeshed *stratum granulosum*.

Sample 3*a*. A small portion from the pyloric caeca and duodenum of fish A was preserved in alcohol and on extraction gave 3·28 % of oil, containing about 20 % of vitamin A:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 70 \\ 617\ \text{,,} & 1000 \\ 583\ \text{,,} & 530 \end{cases}$$

corresponding to some 6600 p.p.m. in the tissue.

Sample 3*b*. The bulk sample corresponding to the above yielded 3·1 % of oil containing about 29 % vitamin A:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 122 \\ 617\ \text{,,} & 1446 \\ 583\ \text{,,} & 735 \end{cases}$$

or about 9000 p.p.m. in the tissue.

Sample 3*c*. The coat from the ileum of fish A gave 1·8 % of oil containing 18 % of vitamin:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 56 \\ 617\ \text{,,} & 903 \\ 583\ \text{,,} & 490 \end{cases}$$

or some 3240 p.p.m.

Sample 3*d*. The coat from the combined intestines of fish B yielded 4·8 % of oil containing 28 % vitamin A:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 70 \\ 617\ \text{,,} & 1400 \\ 583\ \text{,,} & 717 \\ 325\ \text{,,} & 432 \end{cases}$$

or 13,440 p.p.m. in the tissue.

Muscle coats. After peeling off the previous coat it was possible to separate with a knife the muscle from the *serosa*. The muscle tissue is not effectively disintegrated by grinding with sand and, as the fat content is low, is best decomposed by refluxing with alcoholic potash.

Sample 4*a*. Using a small sample from the pyloric caeca and duodenum of fish A, preserved in alcohol, the non-saponifiable extract (0·23 %) was tested:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 16 \\ 617\ \text{,,} & 220 \\ 583\ \text{,,} & 135 \end{cases}$$

The vitamin content of 4·5 % corresponds to *c*. 103 p.p.m. in the tissue.

Sample 4*b*. The main bulk of the above material was worked up at once without treatment with alcohol. About 1·1 % of oil was obtained containing 2 % of vitamin A, or 220 p.p.m. of vitamin A in the tissue.

Sample 4*c*. The muscle coats from the ileum of fish A yielded 1 % of oil containing *c*. 0·5 % of vitamin A. The estimated amount lay between 50 and 70 p.p.m.

Sample 4*d* consisted of muscle from the combined intestines of fish B and was decomposed with alcoholic potash. The non-saponifiable matter (0·33 %) gave the following data:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 13{\cdot}4 \\ 617\ \text{,,} & 283 \\ 583\ \text{,,} & 147 \\ 325\ \text{,,} & 91 \end{cases}$$

corresponding to 5·65 % or 187 p.p.m.

Serosa. This was always carefully freed from adhering mesentery.

Sample 5*a* (12·4 g.), from the pyloric caeca and duodenum of fish A preserved in alcohol, was treated with alcoholic potash and gave 0·3 % of non-saponifiable matter:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 36 \\ 617\ \text{,,} & 470 \\ 583\ \text{,,} & 254 \end{cases}$$

containing *c*. 9·4 % vitamin A or 282 p.p.m. in the tissue.

Sample 5*b*. A bulk extraction of the above material gave 1·2% of oil containing 3·5% of vitamin, or 420 p.p.m.

Sample 5*c*, from the ileum of fish A, contained 1·55% of oil of which about 2% was vitamin A. This corresponds to 310 p.p.m.

Sample 5*d*, from the combined intestines of fish B, was saponified directly and yielded 0·48% of non-saponifiable matter containing 7% of vitamin A, or *c*. 339 p.p.m. in the tissue.

Mesentery. The mesenteric tissue adhering to the gut was also examined.

Sample 6*a*, composed of tissue from the entire intestines of fish A, was saponified after preservation in alcohol and gave 0·4% of non-saponifiable matter:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & 49 \\ 617\ \text{,,} & 693 \\ 583\ \text{,,} & 404 \end{cases}$$

containing about 14% of vitamin A or *c*. 560 p.p.m. in the tissue.

Sample 6*b*. The corresponding bulk sample yielded 1·25% of oil with *c*. 5% of vitamin A, or 625 p.p.m. in the tissue.

Sample 6*c*. The mesentery from fish B yielded 2·2% of oil:

$$E^{1\%}_{1\,\text{cm.}} \begin{cases} 693\ \text{m}\mu & \text{Feeble} \\ 617\ \text{,,} & 169 \\ 583\ \text{,,} & 90 \\ 328\ \text{,,} & 53 \end{cases}$$

containing 3·25% of vitamin, or 734 p.p.m. in the tissue.

The data are collected for reference in Tables I and II, the most probable values being given.

Table I. *Data for fish A*

	Pyloric caeca and duodenum			Ileum		
Coat	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.
Mucosal epithelium*	920	2·2	2,680	—	—	—
Tunica propria	235	11·0	33,000	38	11·0	35,750
Stratum compactum / *Stratum granulosum*	164	3·1	9,000	61	1·8	3,240
Muscle	105	1·1	220	32	1·0	70
Serosa	124	1·2	480	27	1·55	310
Mesentery*	419	1·25	625	—	—	—

* Material from the combined pyloric caeca, duodenum and ileum.

Table II. *Data for fish B*

	Combined pyloric caeca, duodenum and ileum			
Coat	Total wt. of tissue g.	Fat %	Vitamin A p.p.m.	Total vitamin A g.
Mucosal epithelium	468	7·5	2,590	1·21
Tunica propria	232	10·3	18,540	4·30
Stratum compactum / *Stratum granulosum*	101	4·8	13,440	1·36
Muscle	92	1·2	187	0·017
Serosa	67	1·6	339	0·022
Mesentery	197	2·2	734	0·144

It is important to know how much of the vitamin A located in the intestines exists as free alcohol and how much as ester. The only satisfactory approach to

this question is to determine the elimination curve of vitamin A in the process of molecular distillation. For this experiment to be worth while, a relatively large quantity of oil must be accumulated from fresh tissues in which autolysis has not occurred to any great extent. Our first attempts to inhibit autolysis (by storage and transport in tins surrounded by ice and salt) have not been successful and at this stage we shall confine ourselves to reporting the free fatty acid contents of various preparations:

Extract from mucosal epithelium	21·0%	F.F.A.
,, *tunica propria*	7·5%	,,
,, *tunica propria* (ileum)	5·3%	,,
,, *stratum compactum* and *stratum granulosum* ...	13·6%	,,

It is desirable to obtain such data on preparations from absolutely fresh tissue in order to distinguish between post-mortem hydrolytic products and such free acid as may exist in the naturally functioning tissue.

Discussion

The outstanding experimental finding is that the vitamin A occurring in the intestines of the halibut is sharply localized in certain coats. In fish A the *tunica propria* is far richer than the other mucosal coats, whilst the latter in turn are much richer in vitamin than the muscle and *serosa*. In fish B the separation is not quite so sharp, but here also the *tunica propria* is the tissue having the highest concentration of vitamin A.

In an earlier paper [Edisbury *et al.* 1938] the view was advanced that vitamin A might assist in absorption processes (especially of fat), in view of its localization in the absorptive portions of the intestine and its relative absence from the rest of the alimentary tract. The bearing of the present results on this hypothesis may now be considered.

The parallelism between the presence of vitamin and of fat in the various tissues, noted for the alimentary tract as a whole, can be seen to hold equally well in the various intestinal coats. Even when account is taken of the fact that the "fat" percentages recorded include vitamin A esters it is true that the glycerides and the vitamin A esters exhibit parallel distributions.

Greene [1913], again working with the king salmon, has shown that the *tunica propria* and the *stratum compactum* are of peculiar significance in the process of fat absorption in fish. The intestine of a fish is less complex than that of a mammal and fat absorption appears to proceed firstly by diffusion from the lumen into the mucosal epithelium. Possibly bile acids assist in making fatty acids water-soluble, as is the current conception in the case of mammals [Verzár & McDougall, 1936; Frazer, 1938], but absorption by villi and the lymph system does not seem to occur.

In the epithelium the fat appears as droplets which stain as neutral fat. The next stage in absorption is the temporary storage of this fat in the spaces of the *tunica propria*, which acts as a kind of temporary reservoir. The *stratum compactum* forms a definite and continuous boundary to this reservoir. "It is a continuous membrane with no discernible openings other than at the points where blood vessels enter. Any fat passing through the *stratum compactum* would have to pass through in solution or else be carried within in the lumen of the blood vessel. In either case no definite fat globules as such get by this membrane from the *tunica propria*."

Thus, the fat globules in the *tunica propria* must be dispersed and rendered diffusible through the walls of the blood vessels or else through the *stratum*

compactum itself. It is noteworthy that fat leaves the *tunica propria* relatively slowly. It is unlikely that bile acids are present in the *tunica propria* and that fatty acids are rendered water-soluble again for diffusion. It is, however, conceivable that the large quantities of vitamin A present in these tissues may make possible the diffusion of fatty acid either through the blood vessel wall or through the *stratum compactum*.

There is evidence that vitamin A may form a complex with protein, as do certain carotenoids (e.g. astacene). Such a complex exists in visual purple and probably also in liver [Edisbury *et al.* 1938]. Such complexes may well form an integral part of the cell structure of these mucosal tissues and the vitamin A, by repeated esterification and hydrolysis, could thus assist in the transport of fatty acid.

With the evidence at present available it would be unwise to speculate further but should later evidence support this hypothesis there will still be many points to be cleared up. It will be necessary to know whether the fat leaves the *tunica propria* by the blood vessels or directly through the *stratum compactum*. The presence of large quantities of vitamin in the latter coat itself (as well as of appreciable, if much smaller amounts in the muscle, *serosa* and mesentery) suggests that at least a part of the fat may diffuse directly through the *stratum compactum*. It would also be of interest to know whether fat is transported to the liver as vitamin esters or as reformed glycerides. In those fish which do not have large quantities of vitamin A in the intestines, some other substance (e.g. cholesterol or phosphatide) may fulfil a similar function. The hypothesis we have tentatively advanced accounts for the presence of very large amounts of vitamin A in the alimentary tract of certain fishes and also suggests one way in which fat may be transported through the intestinal wall.

Summary

The large vitamin A deposits in the intestines of many species of fish are shown by two methods, applied to halibut, to be sharply localized in the *mucosa* and in particular in the *tunica propria*.

The distribution of fat in the intestine runs parallel with that of vitamin A.

Absorption of fat by fish differs from that obtaining in mammals, proceeding directly through the mucosal epithelium and not by a lymphatic system. The existence of a dense, continuous layer of connective tissue (*stratum compactum*) forms a barrier to fat transport as droplets, which accumulate in the adjacent *tunica propria*.

The hypothesis is advanced that vitamin A esters, in combination with protein, may assist in the dispersion of these droplets and in the removal of fat from the *tunica propria*.

Grateful thanks are due to the Medical Research Council for a grant and to Mr R. H. Creed for technical assistance.

REFERENCES

Edisbury, Morton, Simpkins & Lovern (1938). *Biochem. J.* **32**, 118.

Frazer (1938). *Analyst*, **63**, 308.

Greene (1912). *Bull. U.S. Bur. Fish.* **32**, 73, Document 777 (1913).

—— (1913). *Bull. U.S. Bur. Fish.* **33**, 149, Document 802 (1914).

Lovern, Edisbury & Morton (1937). *Nature, Lond.*, **140**, 276.

MacConaill (1937–8). *J. Anat., Lond.*, **72**, 177.

Verzár & McDougall (1936). Monographs on Physiology: Absorption from the Intestine. London: Longmans, Green & Co.

XLII. HALIBUT INTESTINAL OIL

By J. A. LOVERN, T. H. MEAD and R. A. MORTON

From the Torry Research Station, Aberdeen, the British Drug Houses, London and the University of Liverpool

(*Received 27 January 1939*)

Halibut intestines have been found to autolyse very rapidly. The fat present in any but the freshest material may therefore have undergone considerable hydrolysis. Oil from stale tissue contains more free acid than that from comparatively fresh material, but so far neutral oils have not been obtained. Hitherto, it has not been possible to carry out extraction sooner than 2 or 3 days after the death of the fish. Preservation in ice and salt certainly slowed down autolysis, but special experiments will be necessary before the free fatty acid content of the functioning intestine can be determined.

A beginning has been made towards ascertaining, (*a*) the proportions of free and combined vitamin A, (*b*) the nature of the fatty acids present in the vitamin esters, (*c*) the relative speeds of enzymic hydrolysis of vitamin esters and glycerides and, (*d*) the extent to which autolysis results in contamination of the "fatty" extract.

The method of molecular distillation applied to oils [Hickman, 1937, 1] permits the approximate determination of free and combined vitamin A provided the oil is not excessively acid and some 100 ml. are available. The use of constant yield oil as solvent [Hickman, 1937, 2] extends the method to small quantities of solute.

In 1937 a sample of oil extracted from the ileum of a relatively fresh halibut was subjected to analytical distillation using Hickman's constant yield oil. 0·5 g. was diluted with 75 ml. constant yield oil and 75 ml. of residue oil from a previous molecular distillation. Fractions were collected (10° temperature and 18 min. time intervals) and vitamin yields were determined by assaying the whole series. About 4 % of the total vitamin A occurred in the fractions collected over the range 90–140° and 96 % was volatile at higher temperatures. The elimination curve over the range 150–240° was tall and narrow with a maximum near 210°. The results indicate that approximately 95 % of the vitamin occurred as esters and that the acids which were combined in the greater part of these did not differ much in molecular weight.

An attempt was made in the autumn of 1937 to collect sufficient halibut intestines at Aberdeen for a distillation of the undiluted intestinal oil. Unfortunately, the material as received from the fishing vessels had already undergone considerable autolysis, so that the oil obtained (150 g.) had a high acid value (92·4).

A trial distillation gave the results shown in Table I.

About 20–30 % of the vitamin existed in the free state and the free acids accompanied the vitamin in the distillates. The high acid content facilitated cyclization and made it difficult to collect the distillates without overheating the condenser.

Accordingly, the remainder of the oil was washed with aqueous K_2CO_3, the oil being first dissolved in ether and the amount of K_2CO_3 calculated from the

Table I. *Molecular distillation of 53 g. halibut intestinal oil. B.V. 8300. Acid value 92·4*

Fraction	Temp. °C.	Wt. (g.)	B.V.	Acid value	Colour test $E^{1\%}_{1\,cm.}$ 620 mμ	Colour test $E^{1\%}_{1\,cm.}$ 580 mμ	U.V. absorption $E^{1\%}_{1\,cm.}$ 325 mμ
1	100–120	8·2	6400	156	454	230	113*
2	130–140	12·6	6200	129	400	210	98†
Residue	—	32·2	9000	47	715	365	204‡

* Fine structure due to cyclization very marked.
† Fine structure less marked.
‡ No fine structure.

acid value. The acid value was reduced from 92·4 to 37 and although a lower acidity would have been desirable the oil was distilled at this stage lest further washing with alkali should change the proportion of free to esterified vitamin.

85 g. of washed oil mixed with 15 g. of residual oil were distilled and 15 fractions were collected:

Table II. *Molecular distillation of washed halibut intestinal oil. B.V. 17,000. Acid value 37*

Fraction	Temp. °C.	Wt. g.	Carr-Price B.V.	U.V. absorption $E^{1\%}_{1\,cm.}$ 325 mμ	Colour test, $E^{1\%}_{1\,cm.}$ 620 mμ	580 mμ	693 mμ	Non-sap. %	Recovered acids %	Mol. wt.
1	90	2·8	17,300	200*	875	480	<50	—	—	—
2	100	4·4	17,300	280*	1185	616	<47	—	—	—
3	110	4·2	17,700	188†	880	490	<42	—	—	—
4	120	5·0	14,250	255†	1100	577	<58	—	—	—
5	130	4·7	10,500	180†	725	400	<43	—	—	—
6	140	5·2	6,380	112·5	337	172	—	53·5	46·9	316
7	150	3·6	6,120	116·6	306	160	—	65·6	37·45	315
8	160–70	7·6	15,676	300	1140	627	—	—	—	—
9	180	3·1	29,000	500	1900	986	93	—	—	—
10	190	2·4	33,300	530	1880	1000	97	55·5	32·8	276
11	200	4·1	29,000	590	2080	1075	113	51·1	45·6	280
12	210	4·7	33,300	550	2006	1060	125	50·7	45·9	278
13	220	4·3	31,500	482	1875	990	93	48·3	51·25	284
14	230–40	9·4	17,300	440	1200	676	58	48·0	52·3	291
Residue	—	34·5	2,900	90‡	211	156	13·4	28·7	68·05	301

* Fine structure marked. † Feeble fine structure. ‡ Anomalous absorption.

From Table II it would seem that about 26 % of the total vitamin A in this sample of oil occurred as free alcohol. The ratio $E^{1\%}_{1\,cm.}$ 325 mμ/620 mμ (ultraviolet absorption to absorption for the main band in the colour test) is curiously variable. For the richest preparations of vitamin A prepared by saponification but not subjected to molecular distillation the ratio is about 0·32 and for the purest distillates of the free vitamin perhaps 0·30. In the richest undistilled esters which we have prepared from intestinal oils (60–70 % vitamin esters) this ratio varies from 0·29 to 0·32.

Now the cyclization product of vitamin A is itself chromogenic towards $SbCl_3$ and it is striking that for fractions 1–5 the ratio $E^{1\%}_{1\,cm.}$ 325 mμ/620 mμ varies from 0·21 to 0·25. The cyclized product may well account for the main discrepancies. Fractions 6 and 7 are the first to show no spectroscopic evidence of cyclized vitamin A, and the ratio jumps to 0·33 and 0·38. Fractions 8 and 9 show an unusually low ratio for vitamin esters, whilst Fractions 10, 11 and 12

are much more nearly normal. It is interesting that much of the non-saponifiable other than vitamin A distils mainly between 140° and 180°. The discrepancies may indicate variable proportions of stereoisomerides of vitamin A in the different fractions, but other explanations are equally plausible.

A similar distillation carried out on a fish liver oil affords a useful comparison:

Table III. *Molecular distillation of a sturgeon liver oil**

Fraction	Temp. °C.	Vitamin A %	Ratio $E^{1\%}_{1\,cm.}$ 325 mμ / 620 mμ	Vitamin esters %	Non-sap. %	Recovered acids %	Recovered acids Mol. wt.	Cyclized vitamin A %	Non-sap. other than vitamin A and spurious A %
Original oil		7·9	0·305	—	24·7	69·5	278·5	None	17
1 (not retained)									
2	100	11·2	0·26	—	27·4	65·0	266	13–14	*c.* 16
3	110	15·1	0·26	—	34·7	57·7	272	<14	*c.* 19·6
4	120	15·8	0·26	—	38·5	56·0	273	<10	*c.* 23
5	130	16·4	0·30	—	43·5	52·0	277	Very little	27
6	140	14·3	0·29	—	49·8	44·8	278	None	35·4
7	150	11·5	0·31	21	55·0	38·7	287	,,	43·5
8	160	9·7	0·30	19	67·7	33·1	293	,,	58
9	170	11·8	0·30	23	64·1	33·8	297	,,	52·2
10	180	21·4	0·30	40	64·5	37·2	280	,,	43·1
11	190	29·5	0·28	57	56·1	44·7	276	,,	27
12	200	31·6	0·29	60	51·4	50·8	273	,,	20
13	210	29·0	0·28	55	47·8	51·3	270	,,	18·8
14	220	29·4	0·29	42	44·2	57·2	279	,,	20·9
15	230	15·9	0·31	30	39·5	61·2	278	,,	23·6
16	240	9·1	0·30	17·5	32·9	66·4	277	,,	23·8
17	250	4·7	0·31	*c.* 9	25·5	72·3	272	,,	20·8
Residue		Very low							

* Part of the vitamin A esters had probably been hydrolysed in the process of extraction.

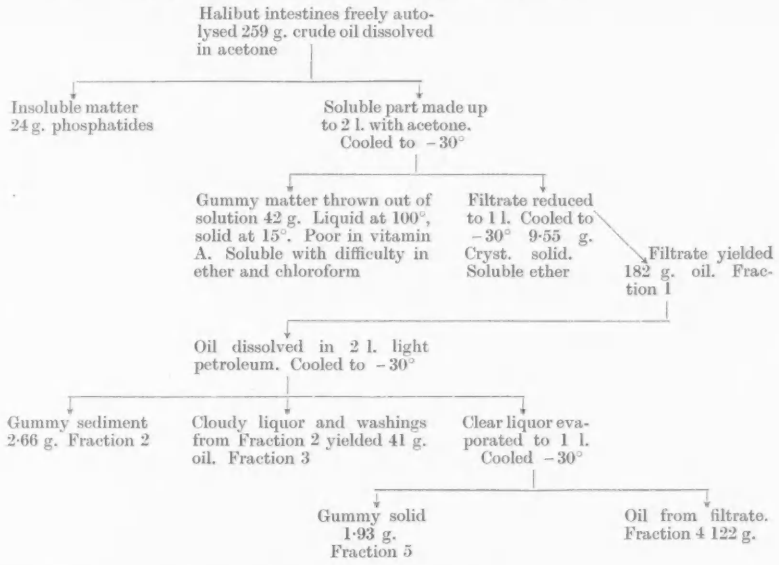
These results indicate a very constant ratio between ultraviolet absorption and colour test intensities once the free acids and cyclized vitamin A have been eliminated. They also illustrate very clearly the limitations imposed by the potency of the original oil and its high content of sterol and sterol esters. Fractions containing 60% of vitamin esters are obtained but the overlap which exists between the elimination curves of vitamin esters and sterol esters sets a limit to the efficacy of the process. The chances of preparing richer ester fractions would be greatly enhanced if sufficient neutral intestinal oil of high potency (60–70% vitamin esters as already described) could be obtained. Unfortunately, the bulk of the halibut recently accessible to us has consisted mainly of smaller fish and it has been impossible to obtain fresh intestines. The next step will be to arrange for better preservation on the fishing vessels.

In the meantime experiments have been carried out on the material available.

Table IV. *Fractionation of oil from autolysed intestines*

Fraction	Non-sap. %	Recovered acids %	Recovered acids Mol. wt.	Colour test, $E^{1\%}_{1\,cm.}$ 693 mμ	Colour test 620 mμ	Colour test 580 mμ	Ultraviolet, $E^{1\%}_{1\,cm.}$ 325 mμ	Ultraviolet 261 mμ
1	21·7	61·7	292	31·5	525	283	158·3	—
2	—	—	—	6·93	110	67·5	38·2	52
3	22·3	65·1	297	29·6	490	286	158	—
4	22·1	70·2	297	32·9	537	302	158·3	—
5	—	—	—	6	27·5	19	11·7	26·6

The following diagram illustrates the scope of the work:


Halibut intestines freely autolysed 259 g. crude oil dissolved in acetone
Insoluble matter 24 g. phosphatides
Soluble part made up to 2 l. with acetone. Cooled to −30°
Gummy matter thrown out of solution 42 g. Liquid at 100°, solid at 15°. Poor in vitamin A. Soluble with difficulty in ether and chloroform
Filtrate reduced to 1 l. Cooled to −30° 9·55 g. Cryst. solid. Soluble ether
Filtrate yielded 182 g. oil. Fraction 1
Oil dissolved in 2 l. light petroleum. Cooled to −30°
Gummy sediment 2·66 g. Fraction 2
Cloudy liquor and washings from Fraction 2 yielded 41 g. oil. Fraction 3
Clear liquor evaporated to 1 l. Cooled −30°
Gummy solid 1·93 g. Fraction 5
Oil from filtrate. Fraction 4 122 g.

Attention may be called to the following points:

(1) The original extract contained about 25% of material which was not glyceride, nor vitamin ester, nor even mainly sterol ester.

(2) Even after the acetone treatment further small quantities of material containing relatively little vitamin can be eliminated.

(3) The process, however, is still far from complete. Thus, the "non-sap." and the acids recovered show a large deficit from 100% in Fraction 1. This is diminished in Fractions 3 and 4 (Table IV).

(4) The gummy residues exhibit a new and well defined absorption band at 261 mμ (Fractions 2 and 5) which is not shown and may be masked in the other fractions. The new absorbing material remains in the aqueous liquor after acidifying the soaps with H_2SO_4 and extracting with ether. It can be extracted from the evaporated residue by means of alcohol but is accompanied by other products.

The whole problem of the occurrence of substances other than glycerides, vitamin esters and sterol esters in "oils" is under investigation and it seems wise to defer the more detailed discussion of the problems raised above. The molecular distillations provide data for constructing elimination curves for vitamin A and vitamin A_2 [cf. Hickman, 1937, 1, 2].

Fig. 1 shows $E^{1\%}_{1\,cm.}$ plotted against temperature for the ultraviolet absorption. The elimination curve shows two maxima, one corresponding with the free vitamin and the other with the esterified vitamin. Fig. 2 shows similar curves for the 693, 620 and 583 mμ bands in the colour test. It is true that the oil contains much more vitamin A than vitamin A_2 and that the proportions are not altogether favourable for a separation. Nevertheless, the similarity between the elimination curves for the two vitamins is very striking and is a sign that the

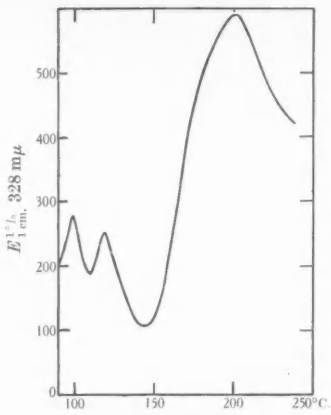


Fig. 1.

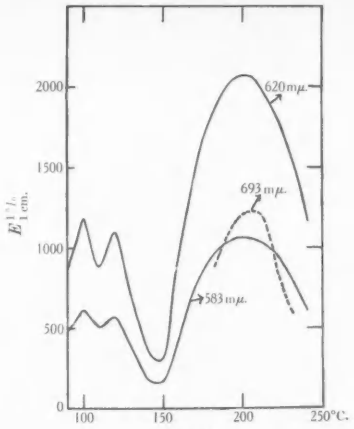


Fig. 2.

Fig. 1. Halibut intestinal oil. The potency of each molecular distillate as measured by U.V. absorption has been plotted against the temperature of distillation. The smaller maxima are due to free vitamin A and the cyclization product, the large maximum to esterified vitamin A.

Fig. 2. $SbCl_3$ colour test data for halibut intestinal oil distillates are plotted against temperature. The 693 mμ maximum concerns vitamin A_2, the 620 mμ and 583 mμ maxima vitamin A.

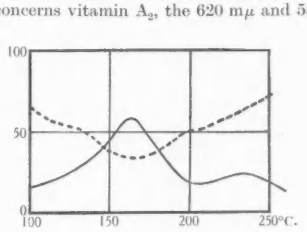


Fig. 3. The non-saponifiable fractions and the acids recovered from the soaps of halibut intestinal oil distillates were obtained and vitamin A estimated spectrographically. — % non-sap. other than vitamin A. --- % recovered acids.

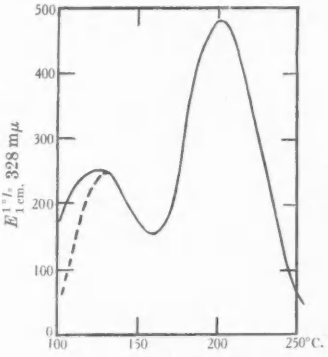


Fig. 4.

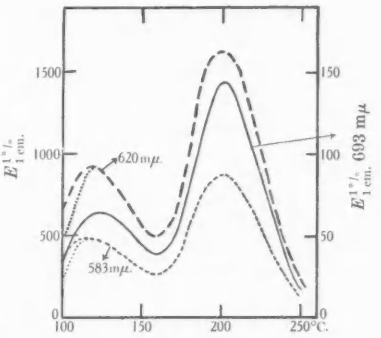


Fig. 5.

Figs. 4 and 5 are comparable with Figs. 1 and 2 and apply to sturgeon liver oil distillates. The dotted portions indicate the course of the curve if no cyclization had occurred.

structure of vitamin A_2 cannot be very different from that of vitamin A. The similarity between the 620 mμ and 583 mμ bands, which both appear to be due to vitamin A, persists after molecular distillation. The elimination curves indicate that both bands are mainly if not entirely due to vitamin A. The halibut intestinal oil which was distilled contained more of the cyclized vitamin than the liver oil used. Cyclization, which is presumably caused by the heating process, is greatly accelerated in the presence of free fatty acid. The elimination curves show quite clearly the extent to which free vitamin A and its cyclization product can be separated. Most of these findings confirm the work of Hickman and his associates. The failure to separate vitamin A from vitamin A_2 is surprising. The work should be repeated on the liver oil from freshwater fish in which vitamin A_2 predominates over vitamin A, and the conditions may prove favourable to the detection of the small differences in the elimination curves if the two vitamins are homologous.

Summary

1. Vitamin A in halibut intestinal oil exists almost entirely (at least 95%) as esters.

2. Halibut intestines autolyse freely so that the extractable "oil" is a mixture of free fatty acids, free cholesterol and free vitamin A with their esters. The oil is also contaminated with phosphatides and other products. Among these is a water-soluble material showing selective absorption λ_{max} 261 mμ. The study of this substance is being pursued.

3. It is difficult to remove free acids from the autolysed material and cyclization occurs to an appreciable extent on distillation.

4. The role of impurities in affecting the physical assay of vitamin A in rich materials is illustrated and, to some extent, accounted for.

5. The elimination curve of vitamin A_2 appears to resemble that of vitamin A very closely.

Our thanks are due to the Medical Research Council for a grant in aid of the expenses of this work (R. A. M.), and to Dr F. H. Carr of British Drug Houses for granting facilities for the molecular distillations.

REFERENCES

Hickman (1937, 1). *J. Industr. Engng Chem.* **29**, 968.
—— (1937, 2). *J. Industr. Engng Chem.* **29**, 1107.

XLIII. THE PHYSIOLOGICAL PROPERTIES OF ASCORBIC ACID

II. THE INCREASE IN METABOLISM OF GUINEA-PIGS ON A DIET DEFICIENT IN ASCORBIC ACID

By EDWARD FIDLAR, MARGARET SHEPPARD and EARL WILLARD McHENRY

From the Department of Physiology and School of Hygiene, University of Toronto

(*Received 23 January 1939*)

It was reported [McHenry *et al.* 1938] that, when two similar groups of guinea-pigs were pair-fed, one group receiving ascorbic acid while the other did not, there was a significant loss in weight in the deficient group at the end of three weeks. In seeking an explanation for this loss in weight, the metabolic rates of the two groups were investigated.

Although the basal metabolism of guinea-pigs suffering from scurvy has been determined by several investigators, conflicting results have been obtained. Söderström & Törnblom [1933], Soneda & Kato [1934], Törnblom [1934], Ardy & Bellini [1936], Fujino [1936] and Scoz *et al.* [1937] all reported that the O_2 consumption in experimental scurvy decreased until death. On the other hand, Mosonyi & Rigo [1933] and Calcinai & Galigani [1934] found a markedly increased O_2 consumption in scorbutic animals.

Methods

In order that the animals in our experiments might be similarly fed, the technique of paired feeding was employed [McHenry *et al.* 1938]. It consists, briefly, in fixing the quantity of food of the ascorbic acid group by the amount of the basal diet consumed by the scorbutic group. In this manner an isocaloric intake was maintained in the two groups. The minimal survival time of the animals on the deficient diet was 21 days, but the metabolic rate was taken on the 18th day. The composition of the basal diet and the care of the animals were similar to those previously reported. Guinea-pigs receiving ascorbic acid were given 5 mg. per day by mouth, a dose which is adequate for growth.

For the determination of the gaseous exchange the respiration apparatus used was that of Macleod *et al.* [1929] with some recent modifications. Although originally planned for dogs it could be used for a group of small animals such as rats and guinea-pigs where the combined volume of the gaseous exchange was sufficiently great to minimize the error. There was no method for recording the activity of the small animals, but in some earlier work with rats it had been found that in a run of about 6 hr. following a 16-hr fast, an average of the two lowest hourly rates gave a figure suitable for comparative purposes even though no record of activity was obtained. It was not always convenient to have the temperatures at that of "thermal neutrality" for the small animals, but when groups were to be compared the average temperature of the cabinet was within a fraction of a degree of the same temperature.

Experimental results

Results were obtained in three series of experiments, the first with two groups of 6 and 8 animals each, the second with two groups of 10 animals each and the third with two groups of 10 animals each together with a normal control group of 10 receiving basal diet *ad lib.* plus ascorbic acid. The O_2 consumption is given on a body-weight basis and the heat output calculated on a body-surface basis in the hope of correcting, to some extent, the errors due to weight differences. The formula used for calculating body surface was $S = 8{\cdot}9 \times W^{\frac{2}{3}}$ where W = weight after the metabolism was measured. For the determination of heat production the caloric equivalent of the O_2 consumption was derived from the R.Q. by the usual table. The highest R.Q. was 0·81, the lowest 0·74, but in the majority of the hourly periods the range was between 0·75 and 0·78. There were no significant differences between groups.

Metabolic rates obtained on the three series are given in Table I. Dates of the experiments are given because of the fact that there is possibly an alteration in guinea-pigs from season to season; at least this applies to resistance to infection or to bacterial toxins.

Table I

Date	Group	No. of animals	Sex	Diet	O_2/kg./hr. ml.	Diff. %	Cal./m²/ 24 hr.	Diff. %
26–27 Apr.	Paired	6	2M., 4F.	Basal + ascorbic acid	925	—	755	—
	Scorbutic	8	4M., 4F.	Basal *ad lib.*	1206	+23	941	+19
31 May–1 June	Paired	10	—	Basal + ascorbic acid	979	—	793	—
	Scorbutic	10	—	Basal *ad lib.*	1282	+30	988	+19
	After 40 mg. of ascorbic acid				1192	—	916	—
18–20 Oct.	Normal control	10	7M., 3F.	Basal *ad lib.* + ascorbic acid	1093	—	925	—
	Paired	10	7M., 3F.	Basal + ascorbic acid	1036	—	872	—
	Scorbutic	10	7M., 3F.	Basal *ad lib.*	1366	+24	1096	+20

There is no record of the sex of animals in the second series but, as in the other two, they were mixed, the two groups being identical in this respect. In the second series an effort was made to demonstrate that the rise in metabolic rate was due specifically to a deficiency of ascorbic acid by giving the deficient animals a total of 40 mg. ascorbic acid and repeating the determination. The metabolism was lowered somewhat and, in all probability, had the treatment been continued, would have returned to normal.

In the third series the normal control group shows that the pair-fed animals receiving ascorbic acid have a metabolism slightly below normal but close enough to be taken as normal in comparison with scorbutic ones.

Discussion

These three sets of experiments carried out in two seasons of the year have shown that guinea-pigs on a diet which though deficient in ascorbic acid is otherwise adequate, exhibit a definite increase in metabolism. Since paired feeding was employed, the results are due to a single variable, namely, the absence of ascorbic acid.

The fact must not be ignored, however, that the calculation of a metabolic rate after a loss in weight can, under certain conditions, be quite misleading.

Should the loss in weight be due to decrease of inert substances such as water and fat while active tissue remains the same in all respects, then the metabolic rate, if based on unit of weight, will appear to rise as the weight falls, although the animal is consuming the same amount of O_2 throughout. A rate in terms of surface area tends to reduce this error, and although some workers maintain that there is no causal relationship between surface area and heat production, this method of expression continues to be employed. Nevertheless, since the formulae ordinarily used for obtaining the surface area of laboratory animals are based entirely on weight, the objection remains that there is no distinction between inert material and active tissue, and therefore increases in metabolic rate associated with weight-loss or decrease in rate accompanying a weight-gain should be carefully considered.

If it be assumed, though quite unjustifiably, that the changes in weight are due solely to fluctuations of inert material, then the weights at the beginning of the experiment might be used as the basis of calculation. Even on this assumption the scorbutic groups show increases in metabolic rate of 13, 13 and 12% respectively in the three series of experiments. Also, the ratios of O_2 consumed to the amounts of dry material found in the bodies of the animals at the end of the experiment confirmed the conclusion that the rise in metabolic rate was not due to loss of water. Under these circumstances there is no need to depart from the conventional form of expression of the metabolic rate as calories per square metre of body surface. Comparisons can then be made with other work.

A number of investigators have suggested that there is a relation between the intake of ascorbic acid and the activity of the thyroid gland. For example, Mosonyi [1936] states that the increase in metabolism observed by him is due to hyperthyroidism, but as yet we have no experimental evidence on which to base such a conjecture.

SUMMARY

Guinea-pigs on a basal scorbutic diet show, on the 18th day of this regimen, a greater metabolic rate than do animals with the same food intake but receiving ascorbic acid.

Grateful thanks are expressed to the Banting Research Foundation for a grant to one of the authors (M. S.).

REFERENCES

Ardy & Bellini (1936). *Riv. Patol. sper.* **6**, 139.
Calcinai & Galigani (1934). *Biochem. Terap. sper.* **21**, 227.
Fujino (1936). *Ark. Med. Fakultät Okayama*, **5**, 60.
Macleod, Hearn & Robinson (1929). *J. Lab. clin. Med.* **14**, 1175.
McHenry, Reedman & Sheppard (1938). *Biochem. J.* **32**, 1302.
Mosonyi (1936). *Klin. Wschr.* **15**, 24.
—— & Rigo (1933). *Hoppe-Zeyl. Z.* **222**, 100.
Scoz, Cattaneo & Gabbrielli (1937). *Enzymologia*, **3**, 29.
Söderström & Törnblom (1933). *Skand. Arch. Physiol.* **66**, 67.
Soneda & Kato (1934). *Sei-i-kai-Med. J.* **53**, 172.
Törnblom (1934). *Skand. Arch. Physiol.* **68**, 171.

XLIV. STUDIES ON DIFFUSING FACTORS
II. COMPARISON OF DIFFUSING FACTORS FROM DIFFERENT SOURCES AND PREPARATION OF CONCENTRATES FROM BULL TESTICLE

By JUAN MADINAVEITIA

From the Biochemistry Department, Lister Institute, London, and the Chemistry Department, University of Manchester

(*Received 27 January 1939*)

It has been recently suggested [Christensen, 1938] that kallikrein, the non-dialysable vasodilator principle from pancreas and urine, is a diffusing factor similar to that in mammalian testicle. This suggestion was based on the fact that intracutaneous injections of kallikrein, mixed with a suitable indicator, result in the spread of the latter over a much larger area than in the case of control injections of indicator alone. The areas were compared 24 hr. after injection.

In endeavouring to compare the relative spreading activities of kallikrein preparations and diffusing factor concentrates, some differences between the modes of action of the two substances have been observed. The technique used for the comparison was that recently described [Madinaveitia, 1938] in which haemoglobin is used as an indicator of the area of skin penetrated by the diffusing factor. The characteristic effect of the testicular diffusing factor is the immediate flattening of the blebs formed by intracutaneous injection of the solution. When haemoglobin is present in the injected fluid the visible effect is a rapid increase of the area of skin coloured by haemoglobin (Figs. 1, 2, 3, curve *DF*).

The effect of intracutaneous injections of kallikrein is different. The skin first becomes oedematous at the site of injection and the oedema slowly spreads. The weal takes a long time to disappear. When haemoglobin is present in the injected fluid it slowly diffuses through this oedematous area (Figs. 1, 2, 3, curve *K*). The area of skin over which the haemoglobin spreads eventually becomes equal to, and even larger than, that following injection of testicular diffusing factor. After some hours the oedema disappears leaving a patch of skin coloured by haemoglobin, identical in appearance with that obtained by injection of testicular diffusing factor. Although the final result as regards the spread of haemoglobin is identical in both cases, the ways in which it is obtained are different. It seems therefore that the testicular diffusing factor and kallikrein are not identical in their biological actions. The available data on diffusing factors, other than the testicular one, are mainly based on measurements taken at least 24 hr. after injection. It has been found desirable to establish whether their actions are like that of the testicular diffusing factor or like that of kallikrein. Leech extracts, rattlesnake venom, solutions of diazobenzenesulphonic acid-serum globulin, *Clostridium Welchii* filtrates, *Vibrion septique* filtrates and staphylococcal toxoid behave, in different degrees of intensity, in a manner similar to the testicular diffusing factor (Figs. 2 and 3).

The mechanism by which the diffusing factors induce water to spread so rapidly through the skin tissues is not yet known. This spread is observed when injections are made in the isolated skin of a rabbit (Fig. 4; see also McClean [1931]). This would indicate that the mechanism by which the diffusion of water takes place through the skin tissues is independent of the circulation of

blood through the skin capillaries. A further support for this view might perhaps be found in the fact that the presence of adrenaline or of histamine in the injected fluid does not interfere with the action of testicular diffusing factor. Neither of these substances has any spreading activity.

Purified preparations of diffusing factor are devoid of haemolytic properties [Favilli & McClean, 1934]. This, together with the fact that substances like saponin, bile salts [McClean, 1930], triethanolamine, soap, allylamine and octyl alcohol do not show the characteristic spreading properties of the testicular diffusing factor, indicates that the spreading is not due to a surface tension effect.

It seems that the diffusing factor is able to increase the porosity of the skin tissues. The increase in the diameter of the pores appears to be substantial, since through them can pass particles as large as those of Indian ink.

Most of the available evidence on the chemical constitution of the testicular diffusing factor suggests that it is a protein, or an active constituent intimately associated with a protein carrier. Nevertheless, some earlier observations did not agree with this point of view. In the preparation of concentrates by fractional lead acetate precipitation [Morgan & McClean, 1932; Madinaveitia, 1938] most of the proteins are removed by precipitation with neutral lead acetate, the active material remaining in the filtrate. Since most proteins precipitate under these conditions it seemed doubtful whether the diffusing factor was a protein or not. Another point which did not seem to be in agreement with the diffusing factor being a protein is the fact that some of the preparations made by Aylward [1937] did not coagulate on heating.

The fractionation of aqueous testicular extracts with $(NH_4)_2SO_4$ has shown that these two facts do not exclude the possibility of the diffusing factor being a protein or being intimately associated with one. Only a small amount of active material precipitates at half saturation with $(NH_4)_2SO_4$, and under the conditions used precipitation is not complete at full saturation (Figs. 5 and 6). From a half-saturated solution the $(NH_4)_2SO_4$ is easily removed by dialysis. The resulting solution retains the full activity of the original extract and contains only 1·6% of the N present in the desiccated bull testicle powder. Half saturation with $(NH_4)_2SO_4$, removal of the precipitate and dialysis of the filtrate give a purification of the same order as that obtained by the fractional lead precipitation method.

McClean [1936], working with diffusing factor from bacterial filtrates, found that it was completely precipitated at full saturation with $(NH_4)_2SO_4$; Claude & Duran-Reynals [1937] came to the same conclusion. These authors, before treatment with $(NH_4)_2SO_4$, precipitated the active material with acetone and extracted this precipitate with water. This procedure denatures the proteins; only 1–10% of the original activity is recovered from the acetone precipitate.

Preparations obtained by $(NH_4)_2SO_4$ fractionation do not give a precipitate with lead acetate, basic or neutral. They readily precipitate with other protein precipitants. The peculiar failure of these proteins to precipitate with lead acetate explains the mechanism of the purification of diffusing factor by means of the fractional lead precipitation. Half saturation with $(NH_4)_2SO_4$ and subsequent dialysis of the filtrate yields a solution of proteins which are not precipitated by lead acetate.

The heat-coagulation of purified $(NH_4)_2SO_4$ diffusing factor is greatly dependent on the *p*H at which the solution is heated. Between *p*H 5 and 6 coagulation was observed, but at other *p*H the solution remained clear after heating. This would indicate that the isoelectric point of this preparation is somewhere between *p*H 5 and 6. No indication is given by previous workers

about the *p*H at which they heated their solutions and it might very well be that some of them were not in the optimum range.

The active constituents of crude testicular extracts cannot be separated from proteins by chromatographic analysis. Some of the proteins present in the extracts submitted to adsorption were less readily adsorbed than the active constituents, and others much more. The separation thus achieved was not so complete as that attained by $(NH_4)_2SO_4$ precipitation.

Experimental

Comparison of testicular diffusing factor with kallikrein

As a source of kallikrein the commercial preparation "Padutin" (Bayer) was used. One unit is stated to correspond to 0·003 mg. of a standard preparation. 9 ml. of "Padutin for oral administration" (1 ml. corresponds to 7 units) were concentrated *in vacuo* over H_2SO_4 to 5·5 ml. A thick syrup resulted. In the shaved back of each of a group of three rabbits two injections of 0·3 ml. of each

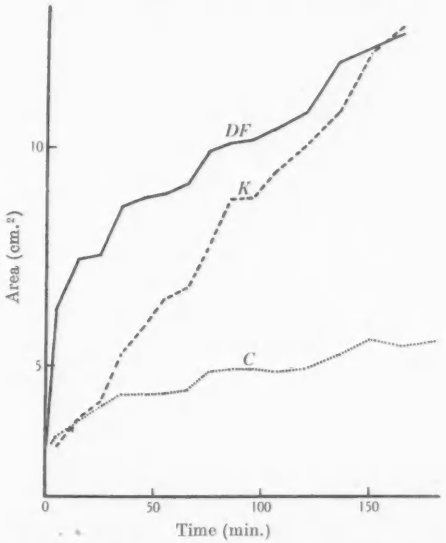


Fig. 1. Comparison of diffusing factors from different sources. *C*, control; *W*, *Clostridium Welchii*; *DF*, testicular diffusing factor; *S*, *Vibrion septique*; *K*, kallikrein; *R*, rattlesnake venom; *T*, *Staphylococcus*; *L*, leech extract; *G*, diazobenzenesulphonic acid-serum globulin.

of the following solutions were made: the concentrated kallikrein solution, a solution of a standard preparation of testicular diffusing factor [Madinaveitia, 1938] having 300 units/ml. and isotonic saline. All three solutions were diluted with 2 vol. of isotonic haemoglobin solution.

Measurements of the size of the coloured areas of skin were made every 10 min. during the first 2 hr. after injection and every 15 min. during the 3rd hr. The size recorded in every instance represents the average of three consecutive readings, the taking of which occupied not more than 5 min. The average of the results obtained in the three rabbits is shown graphically in Fig. 1.

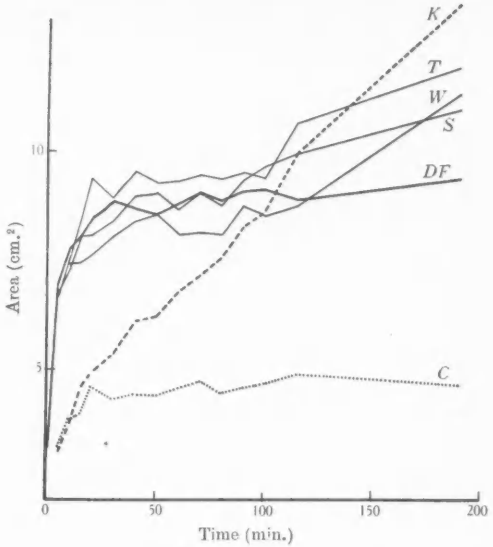


Fig. 2. For description see Fig. 1.

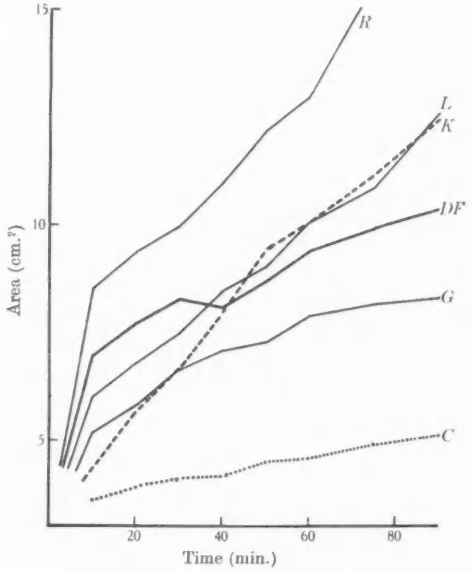


Fig. 3. For description see Fig. 1.

Comparison of diffusing factors from different sources

The bacterial diffusing preparations compared were filtrates of cultures of *Clostridium Welchii*, *Vibrion septique* and *Staphylococcus* toxoid, diluted with 2 vol. of an isotonic solution of haemoglobin. 0·3 ml. of each solution was injected into the shaved back of two rabbits. Injections were also made of 1 : 3 dilutions in isotonic haemoglobin solution of concentrated kallikrein solution (see above), a solution of testicular diffusing factor (100 units/ml.) and isotonic saline. Fig. 2 shows the rate of spread of haemoglobin in each case. The measurements were made as described in the previous experiment.

In another group of three rabbits the following solutions were compared in a similar way: diazobenzenesulphonic acid-serum globulin (kindly supplied by Prof. C. R. Harington) containing about 0·8% protein, a filtered leech extract prepared by grinding up four leeches in a mortar with 25 ml. of water, and a solution of rattlesnake venom containing 30 mg. dry venom per ml. Injections of solutions of diffusing factor (1000 units/ml.) and of a concentrated solution of kallikrein were also made, as well as a control injection of isotonic saline. The average of the results obtained in the three rabbits is represented in Fig. 3.

Action of testicular diffusing factor on the isolated skin of a rabbit

The back of a rabbit was shaved and the animal killed and skinned. The skin was fixed on a board over wet cotton wool. Into this prepared skin injections of 0·3 ml. of serial tenfold dilutions (in isotonic haemoglobin solution) of the

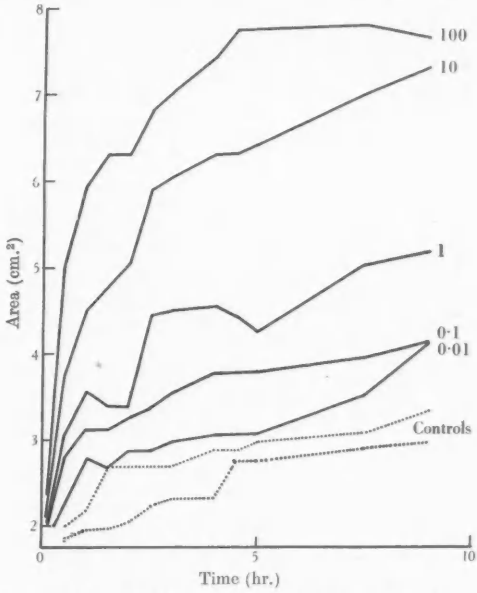


Fig. 4. Increased spread of haemoglobin in the isolated skin of a rabbit.

standard preparation were made. The most concentrated had 100 units/ml. and the most dilute 0·01 unit/ml. Two control injections of saline were also made.

The increase in the area of skin in each case stained by haemoglobin is represented in Fig. 4. Since only one animal was used in the experiment the curves are not so continuous as when the average of results in several animals is represented. The temperature of the skin during the experiment was 18–20°.

Fractionation of aqueous extracts of bull testicle with $(NH_4)_2SO_4$

1 g. of dry testicle powder mixed with the same bulk of silver sand was extracted in a mortar with 20 ml. of water. After centrifuging the turbid supernatant liquor was flocculated by addition of 0·15 ml. 2*N* acetic acid. Centrifuging now yielded a clear solution with the full activity of the original material.

Increasing amounts of saturated $(NH_4)_2SO_4$ were added to this solution as indicated below:

% of saturation	20	33	50	75	90
ml. testicular extract	4	2	2	2	1
ml. sol. sat. $(NH_4)_2SO_4$	1	1	2	2	9

The precipitate in each case was separated by centrifuging and washed with 5 ml. of $(NH_4)_2SO_4$ of the corresponding concentration. Both the precipitates and supernatants of each precipitation were made up to ten times the original volume of testicular extract used. All solutions were diluted with 10 vol. isotonic haemoglobin solution. Their spreading activities were compared with that of serial tenfold dilutions of the testicular extract. A group of two rabbits was used for the precipitates and another group for the supernatants. The average of the results is plotted in Figs. 5 and 6. The precipitate obtained at 50 % saturation with $(NH_4)_2SO_4$ has less than 10 % of the original activity. At 75 and 90 % of saturation the precipitation is still incomplete, both precipitate and supernatant having more than 10 % of the activity of the original solution.

For the preparation of purified concentrates of diffusing factor the following procedure has been used: 250 g. of a dry testicle powder (5 % moisture, 12·5 % ether-soluble materials 11 % N) were extracted overnight in a ball mill with 1 l. of water. A small amount of toluene was added as an antiseptic. After dilution with 1 l. of water 10 ml. 3*N* acetic acid were added and the mixture filtered after 1 hr. The residue was extracted during 4 hr. with 1 l. of water, filtered, and the volume of the united filtrates reduced *in vacuo* at 20° to 250 ml. This concentrated solution contains 58 mg. N/ml. (53 % of the N present in the dry testicle powder). To it 1 vol. of saturated $(NH_4)_2SO_4$ was added and after 1 hr. the precipitate removed. The clear solution was then dialysed against running tap water for 1 day, concentrated *in vacuo* at 20° at about 300 ml. and again dialysed for a day. After making up the volume of the solution to 400 ml. it contained 2·2 mg. N/ml. (i.e. 1·6 % of the N present in the 250 g. dry testicle powder).

The solution so obtained has the full activity of the original testicular extract. It gives all the usual precipitation reactions of proteins, except that it is not precipitated by lead acetate, basic or neutral.

The effect of heat on the coagulation of this solution is recorded in the table below, 2 ml. of solution were heated with 10 ml. of buffer in a water bath.

M/3 acetate buffer of *p*H	3	4	5	6		
M/15 phosphate buffer of *p*H	.	.	.	6	7	8
Temp. ° C.						
70	C	C	C	C	C	C
75	C	C	T	T	C	C
80	C	C	T	T	C	C
90	C	C	T	F	C	C
100	C	C	F	F	C	C

C, clear; T, turbid; F, flocculation.

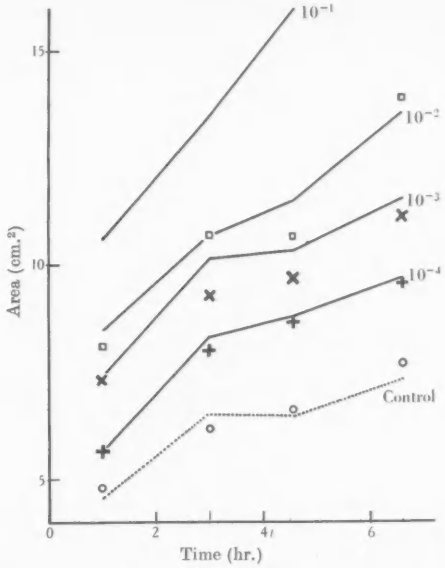


Fig. 5. Fractionation with $(NH_4)_2SO_4$. Activity of precipitates.

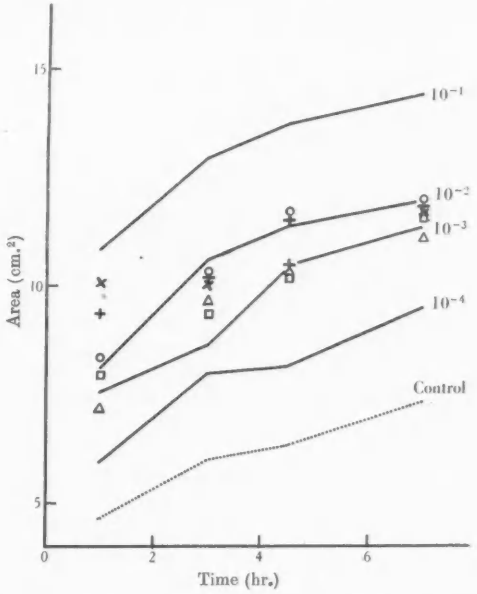


Fig. 6. Fractionation with $(NH_4)_2SO_4$. Activity of supernatants.
○ 20%; + 33%; × 50%; □ 75%; △ 90%

Adsorption of crude testicular extracts on aluminium oxide

250 g. dry testicle powder were extracted (mechanical stirring for 1 hr.) three times with 4 l. of water. The insoluble material was separated each time

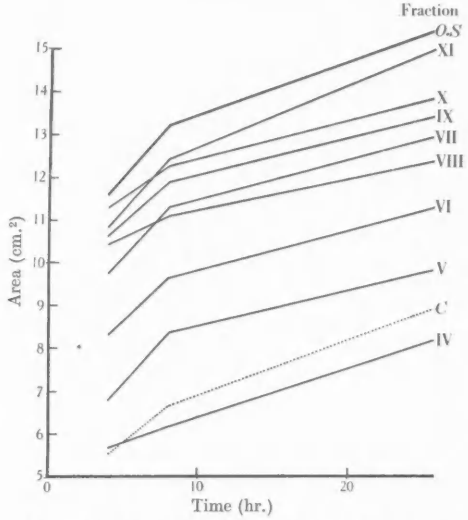


Fig. 7. Adsorption of crude testicular extract on aluminium oxide.

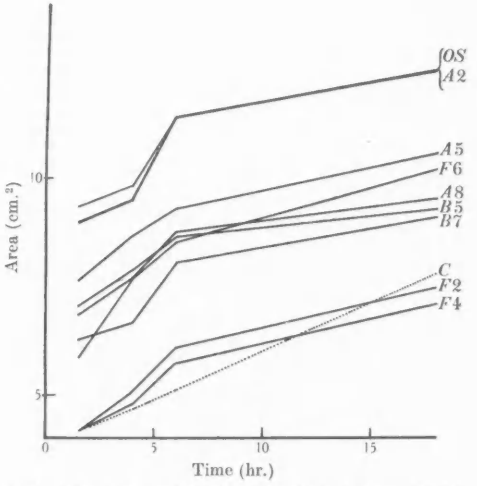


Fig. 8. Adsorption and elution of crude testicular extracts.

by decantation, and the supernatants mixed. To these 100 ml. $N/10$ acetic acid were added and the precipitate allowed to sediment in the cold room. The clear

supernatant was passed through a column (10 × 4·5 cm.) of aluminium oxide (Merck) (100 g.) previously wetted with water. The receiver was changed every 250 ml. (30 min.). After the first four fractions were collected the vacuum was increased and the receiver changed every 500 ml. (20 min.). Two yellowish bands were observed, the first reaching the bottom of the column when 3 l. of solution had passed through. In the table below some of the properties of the resulting fractions are recorded. Tenfold dilutions (in isotonic haemoglobin solution) of fractions iv–xi were injected into the back of three rabbits. The spread of haemoglobin through the skin is recorded in Fig. 7. Fraction vi giving tests for protein has not the full activity of the original solution (*OS*) while fraction ix, although it does not give a precipitate with lead acetate, has almost the same activity as the original solution.

Fraction no.	*OS*	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi
Vol. of fraction in ml.	.	250	250	250	250	550	500	400	500	475	500	600
Vol. of liquid through column in l.	.	0·25	0·5	0·75	1	1·5	2	2·5	3	3·5	4	4·5
Precipitation by trichloroacetic acid	+	−	−	−	−	−	+	+	+	+	+	+
Precipitation by neutral lead acetate	+	+	±	±	±	±	+	+	±	−	+	+
Heat-coagulation	+	−	−	−	−	+	+	+	+	+	+	+
Biuret test	+	−	−	−	±	+	+	+	+	+	+	+

In another experiment a similar column of aluminium oxide (Hopkins and Williams pure anhydrous) was prepared. 300 ml. of the testicular extract used in the previous experiment (2·63 mg. *N*/5 ml.) were filtered through it, changing the receiver every 50 ml. The column was then washed with 400 ml. *M*/500 acetic acid and subsequently with 1% $NaHCO_3$ (400 ml.). The behaviour towards trichloroacetic acid and the N content of some of the fractions collected are shown in the following table.

Filtrates (*F*)

Fractions of 50 ml.	1	2	3	4	5	6
Trichloroacetic acid	−	−	−	+	+	+
mg. *N*/5 ml.	.	0·014	.	0·45	.	0·94

Acetic acid washings (*A*)

Fractions of 50 ml.	1	2	3	4	5	6	7	8
Trichloroacetic acid	+ +	+ +	+	+	+	+	±	±
mg. *N*/5 ml.	.	2·48	.	0·384	.	.	.	0·128

Bicarbonate washings (*B*)

Fractions of 50 ml.	1	2	3	4	5	6	7	8
Trichloroacetic acid	±	?	+	+	±	±	±	?
mg. *N*/5 ml.	.	.	0·331	.	.	.	0·069	

The area of skin over which haemoglobin is induced to spread by injection of 1 : 10 dilutions (in isotonic haemoglobin solution) of some of these fractions is shown in Fig. 8. After most of the active materials have been eluted with acid, substantial amounts of inactive protein are eluted from the column by $NaHCO_3$.

Summary

1. Comparison of testicular diffusing preparations with kallikrein indicate that, contrary to the statement of Christensen [1938], the two materials are quite unlike one another in their effects on the rate of spread of injected fluids through rabbit skin.

2. Rattlesnake venom, filtrates of *Clostridium Welchii* and *Vibrion septique*, staphylococcal toxoid, leech extracts and diazobenzenesulphonic acid-serum globulin all resemble the testicular diffusing factor in producing a very rapid increase in the rate of spread of fluids through skin.

3. Purification of the testicular diffusing factor by means of fractional precipitation with ammonium sulphate and dialysis is described. This method yields products of similar activity to those previously described [Madinaveitia, 1938].

4. The main bulk of evidence indicates that the active material has protein properties and that its biological action, which does not depend on circulation, is not due to a surface tension effect.

The author wishes to express his thanks to Prof. A. R. Todd and Dr D. McClean for their continued interest and encouragement. He is also grateful for the hospitality of the Lister Institute where the early part of this work was carried out. Gifts of bacterial filtrates from Dr D. McClean and of rattlesnake venom from Dr F. Duran-Reynals are gratefully acknowledged.

REFERENCES

Aylward (1937). *Proc. Soc. exp. Biol., N.Y.*, **36**, 477.
Christensen (1938). *Nature, Lond.*, **142**, 37.
Claude & Duran-Reynals (1937). *J. exp. Med.* **65**, 661.
Favilli & McClean (1934). *J. Path. Bact.* **38**, 153.
Madinaveitia (1938). *Biochem. J.* **32**, 1806.
McClean (1930). *J. Path. Bact.* **33**, 1045.
—— (1931). *J. Path. Bact.* **34**, 459.
—— (1936). *J. Path. Bact.* **42**, 457.
Morgan & McClean (1932). *J. Soc. Chem. Ind., Lond.*, **51**, 912.

XLV. THE MECHANISM OF THE FORMATION OF ORGANIC ACIDS BY MOULD FUNGI

III. THE INFLUENCE OF IODOACETATE AND OF FLUORIDE ON THE FORMATION OF ACIDS

By ETHEL MURIEL JOHNSON, EDWIN COULTHARD KNIGHT and THOMAS KENNEDY WALKER

From the College of Technology, University of Manchester

(*Received 31 January 1939*)

The action of *A. niger* in the presence of sodium iodoacetate [Johnson *et al.* 1937] has now been studied with sucrose as the substrate and the experiments have yielded results similar to those found with glucose. Thus $M/450$ iodoacetate inhibited anaerobic formation of ethyl alcohol by six different strains of *A. niger* which were able in the presence of $M/400$ iodoacetate to produce both citric and oxalic acids in quantities sufficient for quantitative estimation. Further, although $M/400$ iodoacetate restricted the average quantity of sugar metabolized in 11 days to about 30%, little difference was noted in the yield of gluconic acid in g. per 100 ml. culture liquid, since the yield of the latter acid expressed as a percentage of the weight of sugar consumed was raised from an average normal value of 13% to a value of 45% in the presence of $M/400$ iodoacetate (Table I).

Also, experiments conducted simultaneously in the presence and in the absence of iodoacetate showed that the latter substance at concentrations of $M/1000$ and $M/500$ caused a large rise in the weight of citric acid and a considerable fall in the weight of gluconic acid produced in unit volume of the culture medium (Table II). After incubation for 20 days in presence of $M/500$ iodoacetate the sugar metabolized was found to be 80% of the quantity present initially, as compared with a consumption of 66·75% in absence of iodoacetate.

Experiments were also undertaken to ascertain the behaviour of *A. niger* towards sucrose in the presence of NaF. Using strain T1 it was necessary to employ NaF at a concentration of $M/100$ in order completely to suppress the formation of citric acid, whereas under anaerobic conditions the formation of ethyl alcohol was inhibited in the presence of $M/200$ NaF. In further experiments with strain T1 conducted aerobically in the presence of NaF at concentrations from $M/200$ to $M/400$ it was found in every case that in 13 days the total acid produced was less than in the "controls" without NaF. The amounts of citric acid formed were particularly low in comparison with those produced in the "control" flasks but, on the other hand, the formation of gluconic acid, which was negligible in the "control" flasks, was strongly stimulated by all concentrations of NaF and reached a maximum when the latter was $M/250$ (Table III). The effects of NaF on acid formation by *A. niger* are similar, in some respects, therefore, to those exercised by iodoacetic acid. Bernhauer [1928] has reported that in the presence of certain concentrations of the commercial germicide "Uspulin" the action of *A. niger* on sugar results in largely increased production of gluconic acid with simultaneous suppression of the formation of citric acid.

The increase of gluconic acid formation in cases such as these may be explained in two ways. The poison may selectively cripple the enzyme system or systems responsible for the formation of citric acid whilst leaving undamaged, or even stimulating, the glucose oxidase which gives rise to gluconic acid. This would leave the glucose oxidase free to transform into gluconic acid the sugar which normally would be converted into citric acid. Alternatively, it is possible that the poison cripples the enzyme system employed by the mould for the conversion of gluconic acid into citric acid. If such is the case the experimental results in question are evidence in favour of the view that gluconic acid is an intermediate in the formation of some, at least, of the citric acid produced from sucrose. Chrząszcz & Tiukow [1930] suggested that the first phase of citric acid formation from sugar under the influence of mould fungi consisted of the processes of alcoholic fermentation, and that this phase was succeeded by one in which acetic acid (formed from acetaldehyde or ethyl alcohol) gave rise to 4-carbon dicarboxylic acids in the following sequence:

$$\text{2 mols. acetic acid} \xrightarrow{-H_2} \text{succinic acid} \xrightarrow{-H_2} \text{fumaric acid} \xrightarrow{+H_2O} \text{malic acid.}$$

The authors presumed that citric acid was then produced by a final dehydrogenation whereby 1 mol. of malic acid was condensed with 1 mol. of acetic acid. Whilst the subsequent work of a number of investigators [Butkewitsch & Gajevskaya, 1935; Wells *et al.* 1936; Clutterbuck, 1936; Walker, 1936] has proved that alcoholic fermentation cannot be an initial phase in citric acid formation, the experiments of Chrząszcz & Tiukow [1930], of Chrząszcz *et al.* [1932] and of Bernhauer *et al.* [1934] have fully substantiated the formation of succinic, fumaric, malic and citric acids in cultures of various moulds in contact with salts of acetic acid or with very dilute ethyl alcohol. Hence, if it could be shown that mould fungi can transform 1 mol. hexose into 3 mol. acetic acid or into 1 mol. acetic acid together with 1 mol. of any one of the above 4-carbon acids, it would then be justifiable to regard the later reactions immediately preceding the formation of citric acid as consisting of dehydrogenations of the type suggested by Chrząszcz & Tiukow. An explanation on these lines would, moreover, approximate very closely to the hypothesis of Raistrick & Clark [1919], who viewed the initial processes of citric acid formation as consisting of dehydration and oxidation of the hexose molecule followed by fission into 1 mol. of acetic acid and 1 mol. of oxaloacetic acid, the two latter substances constituting the immediate precursors of the citric acid to which, by an aldol condensation, they were presumed to give rise. Views similar to those of Raistrick & Clark were also expressed later by Virtanen & Pulkki [1930]. Whilst these assumptions are quite in harmony with the known properties of the several substances involved, neither acetic acid nor oxaloacetic acid has yet been isolated from cultures of *A. niger* on sugar. According to Bernhauer *et al.* [1932] small quantities of malic acid may sometimes be detected in cultures of this mould on sugar solutions, a statement which we can confirm from our own experiments, and possibly this malic acid arises by reduction of oxaloacetic acid. It is equally possible, however, that such malic acid is derived from succinic acid, and this view leads to consideration of the possibility mentioned above, namely, that fission of the sugar molecule under the influence of the mould may give rise to the production of equimolecular quantities of acetic and succinic acids. Support for the belief that a non-phosphorylated glucose molecule can, by reactions involving fission, give rise to succinic acid, is found in the phenomena observed by Virtanen [1925] when investigating cell-free propionic acid fermen-

tation, cf. Virtanen & Simola [1927]. Moreover, Scheffer [1928], working with Kluyver, obtained quantitative data in favour of the conception that in the fermentation of glucose by *B. coli* some of the sugar is split into 4-carbon and 2-carbon compounds. These observations led one of us to suggest [Johnson, 1934] that formation of citric acid may take place through an initial conversion of a molecule of hexose into 1 mol. acetic acid and 1 mol. succinic acid, and that these two acids would then give rise to the final product in accordance with the scheme of Chrząszcz & Tiukow or with one of the closely related schemes suggested by Bernhauer *et al.* [1934]. Alternatively and with equal reason the succinic acid could be assumed to undergo conversion into oxaloacetic acid when the final condensation would be that postulated by Raistrick & Clark.

Gudlet [1936], from consideration of Virtanen's work, has also arrived at the same opinion and has suggested that the conversion of sugar into acetaldehyde and succinic acid, without loss of CO_2 by decarboxylation, is a necessary step in the production of citric acid by moulds.

Facts which have been co-ordinated by Kluyver [1935] would seem to provide a reasonable explanation of the mechanism of the formation of both acetic and succinic acids from glucose by microbiological agency. He has brought forward the view that the formation of these acids from glucose by *B. coli* occurs through an initial fission of the hexose to ethylene glycol and tartaric acid dialdehyde, followed by transformation of the former into acetaldehyde and of the latter into succinic acid. Further, the well known formation of equimolecular quantities of acetic and lactic acids from xylose by the action of *Lactobacillus pentosus* is a conversion which is readily understandable if considered as completely analogous to the case of the action of *B. coli* on glucose, for the xylose may be presumed to yield glycollic aldehyde and glyceric aldehyde which are then transformed into acetic acid and lactic acid, respectively.

Consideration of the citric acid problem from this standpoint has led us to devise further experiments to gain information respecting the processes involved in the production of this acid from sugar. If succinic acid is an intermediate it follows that any agent which serves to inhibit its oxidation might also be expected to exercise an inhibitory effect on the production of citric acid in cultures of *A. niger* on sugar. Accordingly, in view of the fact that we had already shown that $M/500$ iodoacetate does not interfere with the production of citric acid from sugar, we next examined the behaviour of citric acid-forming moulds towards Na acetate in the presence of iodoacetate, and found that at $M/500$ concentration of the latter poison the synthesis of both malate and citrate from acetate by two species of *Penicillium* was not prevented and, in agreement with this result, we also made the observation that $M/500$ iodoacetate did not interfere with the conversion of succinic acid into *l*-malic acid under the influence of *A. niger* (strain N 1). In the presence of $M/450$ iodoacetate *l*-malic acid was still produced, though in diminished yield, from succinic acid. According to Quastel & Whetham [1924] NaF strongly inhibits succinodehydrogenase. Since we had found that NaF at concentration $M/150$ allowed the formation by *A. niger* of a little citric acid from cane sugar, whereas at concentration $M/100$ no citric acid was obtained, the effects of NaF were tried in cultures of *A. niger* on succinic acid. It was found that whereas malic acid was still produced (though only in traces) when the concentration of fluoride was raised to $M/150$, at $M/100$ the formation of malic acid could no longer be demonstrated. It appears, therefore, that the respective concentrations of iodoacetate and NaF which were found to be just sufficient to prevent the mycological conversion of sugar into citric acid are also the concentrations which are necessary to prevent

the conversion of succinate (or acetate) into malate or citrate. Hence, none of the evidence provided by our experimental results disagrees with the suggestions made by one of us [Johnson, 1934] of a possible mechanism of citrate formation from sugar, *via* succinate plus acetate.

Experimental

The moulds employed were six pure strains of *A. niger*, designated N1, N2, B7, B12, B16 and T1, respectively, and cultures of *Penicillium citrogenum* and of *P. Johannioli*. The two last named are citric acid-producing species which were kindly placed at our disposal by Prof. T. Chrząszcz of Poznań. For experimental purposes they were cultivated in most cases on a sterilized medium containing 0·2 % NH_4NO_3; 0·1 % KH_2PO_4; 0·05 % $MgSO_4$, $7H_2O$ and glucose or sucrose, 10 or 12·5 %.

Action of A. niger *on sucrose in presence of* M/*400 Na iodoacetate*

Six strains of *A. niger* were grown at 30° from spores sown on the nutrient salt medium containing 10 % of sucrose in 24 Erlenmeyer flasks of 1500 ml. capacity, each flask containing 300 ml. liquid. After 5 days the sugar solutions were poured away, the mycelia were washed as described [Johnson *et al.* 1937] and a fresh medium consisting of 10 % sucrose containing *M*/400 Na iodoacetate was introduced under the mycelia in 12 of the flasks. The other 12 flasks were recharged with 10 % sucrose without iodoacetate and were used as controls. All the 24 flasks were then returned to the incubator for a further period of 11 days. The analytical figures are shown in Table I.

Table I. *Iodoacetic acid (as Na salt) at concentration* M/*400 in 10 % cane sugar solution*

Incubation at 30° for 11 days

Strain of *A. niger*	Glucose consumed, % of initial wt.	Acidity, ml. 0·1*N* NaOH to neutralize 100 ml. medium	Acid produced, expressed as % by wt. of the sugar consumed		
			Citric acid	Oxalic acid	Gluconic acid
T 1	13	36·3	5·1	0·7	36·3
N 1	14	45·1	3·0	1·7	46·5
N 2	11	30·8	3·3	1·1	40·3
B 12	11	31·9	2·6	1·7	41·3
B 7	14	49·5	3·2	2·1	50·4
B 16	15	59·4	5·5	1·4	55·1
"Controls", in absence of iodoacetate					
T 1	43·5	110	10·7	—	17·0
N 1	66·6	271	9·7	9·1	17·4
N 2	55·6	150	12·6	2·2	5·9
B 12	62·5	213	13·6	1·5	19·3
B 7	56·0	147	11·0	0·6	15·0
B 16	47·8	117	14·2	0·4	2·9

In order to establish beyond doubt the identities of the three acids (presumed to be citric, oxalic and gluconic, respectively) produced in presence of *M*/400 iodoacetate (Table I), they were converted into derivatives. From the two first named, the *p*-nitrobenzyl esters were prepared; they melted at 102 and 204°, respectively, alone or in admixture with authentic specimens. The Ca gluconate was purified [cf. Butkewitsch, 1924] and dried at 100–110°. Found: CaO, 12·9 %. Calc.: CaO, 13·02 %. The phenylhydrazide of the acid was prepared and sintered

at 195°, M.P. (decomp.) *c.* 200° [cf. Butkewitsch, 1924; Fischer & Passmore, 1889]. Found: C, 50·2; H, 6·5; N, 10·0%. $C_{12}H_{18}O_6N_2$ requires C, 50·35; H, 6·3; N, 9·8%.

Action of A. niger *on sucrose in presence of Na iodoacetate* M/*1000 and* M/*500*

The mould T1 was cultivated in 20 flasks of 350 ml. capacity each charged with 100 ml. 10% sucrose saline medium. After growing for 4 days at 30° the mycelia were washed and the medium in 10 flasks was replaced by 10% sucrose and in the other 10 flasks by 10% sucrose together with *M*/1000 Na iodoacetate. This experiment was repeated with another series of 20 flasks, in which case *M*/500 iodoacetate was used. For the analyses, samples withdrawn from each of the 10 flasks which formed a series were mixed; thus the figures represent the averages of ten values in each case. The data are shown in Table II.

Table II. A. niger, *T 1, incubated at 30° on 10% sucrose solution in presence and in absence of* M/*1000 and* M/*500 Na iodoacetate*

Time in days	Acidity, ml. 0·1*N* NaOH to neutralize 100 ml. medium	Acidity expressed as mg. acid per 100 ml. medium			Acidity, ml. 0·1*N* NaOH to neutralize 100 ml. medium	Acidity expressed as mg. acid per 100 ml. medium		
		Citric acid	Oxalic acid	Gluconic acid		Citric acid	Oxalic acid	Gluconic acid
	In presence of *M*/1000 iodoacetate				In absence of iodoacetate			
3	30	+	–	–	62	+	–	–
6	58	+	–	–	95	39	–	1744
8	64	155	+	842	116	78	22	2000
10	72	227	+	725	125	115	46	2000
13	84	266	3	842	158	143	89	2646
15	96	305	5	960	166	127	115	2606
17	106	337	5	1038	174	95	156	2783
22	144	470	202	1352	204	82	237	3234
	In presence of *M*/500 iodoacetate				In absence of iodoacetate			
6	58	216	–	666	86	93	–	1460
8	66	237	–	568	98	96	63	1489
10	74	258	Trace	666	110	98	73	1705
13	96	358	103	686	134	102	101	2097
15	108	340	155	744	158	132	125	2430
17	118	335	193	862	169	145	145	2556
20*	136	238	345	1192	152	102	163	2313

* On the 20th day sugar estimations showed that in the flasks containing *M*/500 iodoacetate the sugar consumption had amounted, on the average, to 80% of the quantity initially present, whilst in absence of iodoacetate 66·75% of the sugar had been consumed.

Action of A. niger *on sucrose in the presence of NaF*

The mould T1 was cultivated on a 10% sucrose medium as in the previous experiments, and on the 5th day from the time of inoculation this medium was poured away, the mycelia were washed, and fresh 10% cane sugar solutions, containing different concentrations of NaF, were introduced under the mycelia in the several flasks. The culture media were examined after further incubation at 30° for 6 days and 13 days, respectively. In Table III the analytical data obtained on the 13th day are given. Oxalic acid was not detected in any of the flasks, and no citric acid could be detected in presence of *M*/100 NaF.

Table III. A. niger, *T 1, incubated at 30° for 13 days on 10% sucrose solution in the presence of NaF*

Conc. of NaF	Sugar consumed, % of initial wt.	Acidity, ml. 0·1*N* NaOH to neutralize 100 ml. medium	Citric acid: (*a*) mg. per 100 ml. medium; (*b*) % of wt. of glucose consumed		Gluconic acid: (*a*) mg. per 100 ml. medium; (*b*) % of wt. of glucose consumed	
			(*a*)	(*b*)	(*a*)	(*b*)
N/150	28	36·3	20	0·7	664	23·6
N/200	28·5	38·5	36	1·3	650	22·9
N/250	34	55·0	62	1·8	900	27·0
N/300	32	48·0	94	2·9	700	24·9
N/400	23	22·0	47	2·0	294	12·8
Control (no fluoride)	45	68·2	422	9·4	44	1·0

The action of Penicillium citrogenum *and of* P. Johannioli on *Na acetate in the presence of* M/*500 Na iodoacetate*

These two *Penicillium* species were chosen in preference to *A. niger* for this particular experiment since they had been found by Chrząszcz & Tiukow to be particularly suitable for demonstrating the mycological production of citric acid from acetic acid. Moreover, previous experiments with *P. citrogenum* had shown that when it was placed on a 12·5% glucose medium production of citric acid was considerably curtailed in presence of *M*/500 iodoacetate.

Cultures of the two moulds were grown in 24 boiling tubes (12 tubes for each species) from spores sown on a medium consisting of sucrose (5%) in nutrient inorganic salt solution. After incubation for 4 days at 25° the mycelia were transferred to 24 conical flasks (1500 ml.) each containing 330 ml. of the same medium and incubation at 25° was conducted for 6 days, after which the mycelial mats were washed thoroughly with sterile water and allowed to stand overnight over water. After this treatment they no longer diffused citric acid into the water. The mycelia were then floated on a sterile aqueous solution of Na acetate (5·6% equivalent to 2·5% acetic acid) containing also *M*/500 Na iodoacetate. The metabolism solutions were collected for examination after the mycelia had been in contact with the acetate medium for 8 days at 25°.

(1) P. citrogenum *cultures*. The mixed culture fluid from the 12 flasks was filtered and evaporated to one-third of its volume after which sat. Pb acetate was added to it in slight excess and the mixture was allowed to stand for 3 hr. The white precipitate which had formed was filtered and washed at the pump until the washings were free from Pb. The filtrate (A) was preserved for examination. The washed precipitate was suspended in 1750 ml. water, 600 ml. 2% acetic acid were added and the whole was warmed for 2 hr. on a steam bath and then filtered. The undissolved solid matter was well washed and the washings were added to the filtrate (B) which was preserved for examination. The washed solid (C) was suspended in water and decomposed by H_2S. The PbS was removed by filtration and the filtrate was evaporated to one-third of its volume. In this liquid were found the largest proportions of acidic products and these were dealt with as follows. Qualitative tests having shown the presence of citric and oxalic acids, these were separated through their Ca salts in the usual manner. A portion of the fraction which was presumed to be Ca citrate after repeated crystallization from hot water and drying at 130° gave 0·2451 g. pure Ca salt. Found: CaO, 33·9%. Calc. for anhydrous tricalcium citrate: CaO, 33·74%. A further portion (1·5 g.) of the same material was treated with the calc. H_2SO_4 and, after removal of the $CaSO_4$, the solution was

evaporated to one-half of its volume, neutralized with 0·5*N* alcoholic KOH and finally evaporated to dryness. To the solution of the K salt in 10 ml. water were added 30 ml. alcohol and 2 g. *p*-nitrobenzyl bromide. The whole was heated under reflux for 2 hr. after which, on cooling, the product separated and was collected and recrystallized from aqueous alcohol. The purified material had M.P. 102° alone or in admixture with an authentic and pure specimen of the tri-*p*-nitrobenzyl ester of citric acid.

The Ca salt obtained from the fraction presumed to contain oxalic acid was purified in the usual manner and analysed. Found: Ca, 38·11%. Calc. for C_2O_4Ca, H_2O: Ca, 38·36%. A small quantity of the acid was converted into its *p*-nitrobenzyl ester which, after purification, had M.P. 204° alone or in admixture with an authentic specimen of the di-*p*-nitrobenzyl ester of oxalic acid.

Filtrate (A) was evaporated to one-half of its volume and this concentrate was mixed with 3 vol. of alcohol, but only a faint cloudiness appeared and no substance could be identified. Filtrate (B) was treated with H_2S, the precipitate of PbS removed and the filtrate evaporated, when a small quantity of sticky solid remained. This was freed from the last traces of water and acetic acid by addition of a mixture of benzene and alcohol and subsequent evaporation *in vacuo*. About 0·5 g. solid material remained which gave a strong positive reaction for malic acid when submitted to Denigès' Hg acetate test. This was confirmed by conversion of the acid into acetaldehyde which in turn was characterized by condensation with dimethyldihydroresorcinol to yield aldomedon, M.P. 142°.

(2) P. Johannioli *cultures*. The metabolism solution from these cultures was treated in a similar manner. Citric acid and oxalic acid were both isolated and characterized as their respective *p*-nitrobenzyl esters, but the quantities of these acids were less than those obtained in the case of *P. citrogenum*.

The action of A. niger *on NaH succinate in the presence of Na iodoacetate*

Seventeen conical flasks (1000 ml.) were each charged with 250 ml. sucrose (5%) in the nutrient salt medium of Wells *et al.* [1936]. After sterilization the solutions were inoculated with cultures of *A. niger*, strain N1, previously developed from spores in boiling-tubes containing the same medium. After incubation for 5 days at 30° the culture fluid was poured away and the mycelial mats were washed with sterile water and allowed to stand for 6 hr. over fresh sterile water. The latter was then poured away and seven of the flasks were each charged with 250 ml. of a sterile 4% aqueous solution of NaH succinate containing *M*/500 Na iodoacetate. Seven other flasks were treated similarly except that in these the concentration of iodoacetate was *M*/450. The remaining three flasks contained succinate without iodoacetate as controls. All the flasks were returned to the incubator at 30° and from time to time samples were withdrawn and tested for the presence of malic acid by Denigès' Hg acetate solution. Positive tests were obtained after 7 days, and after 14 days the solutions were combined into two lots and examined. Since the colour in both cases was too dark to permit direct polarimetric examination, aliquots (100 ml.) of each were made alkaline with strong NH_3 and treated with excess Pb acetate. After the addition of 50 ml. alcohol each solution was allowed to stand for 48 hr. The precipitates which had settled were then filtered and after washing with 30% alcohol they were ground with water to a fine suspension and treated with H_2S. The PbS was removed, the clear solutions were boiled to expel H_2S and

their rotations were measured in 1 dm. tubes in the presence of ammonium molybdate [Williamson, 1918] with the following results: sample from *M*/500 iodoacetate flasks, +1·6° (Ventzke scale); sample from *M*/450 iodoacetate flasks, +0·6°; sample from control iodoacetate flasks, +0·85°. Since molybdate reverses the sign of rotation, these results represent *laevo* rotations caused by *l*-malic acid. A preliminary test had shown that *M*/450 iodoacetate did not affect the accuracy of Williamson's method. Since the separation of Pb salts was not quantitative, the values for the solutions incubated in presence of iodoacetate cannot be compared accurately with the "control".

The action of A. niger *on NaH succinate in the presence of NaF*

Preliminary experiments indicated that spores of *A. niger* (strain N1) would germinate in sugar solutions containing *M*/150 NaF but not in *M*/100. To each of a series of conical flasks (1000 ml.) containing 250 ml. of a 4 % aqueous solution of NaH succinate, a different quantity of NaF was added and three flasks were left as controls without NaF. All were inoculated with spores of *A. niger* (strain N1) and were incubated at 30°. The mould grew freely in all the flasks and after 14 days Denigès' test for malic acid was positive in all cases, but the amounts of malic acid detected, including those found in the control flasks, were very small. After 28 days a mixed sample from the controls was treated with ammonium molybdate for polarimetry in a 2 dm. tube, when the rotation was found to be +0·45° (Ventzke scale) corresponding to a 0·015 % solution of *l*-malic acid, while the culture liquid containing *M*/150 NaF on similar treatment showed +0·3°, corresponding to 0·01 % *l*-malic acid. A preliminary test had shown that *M*/150 NaF did not interfere with the polarimetric estimation of malic acid by this procedure.

Summary

1. The effects of *M*/400 Na iodoacetate on the behaviour of *A. niger* (6 strains) in contact for 11 days at 30° with 10 % sucrose solution, as shown by comparison with cultures incubated in absence of iodoacetate, resulted in (*a*) a restriction in the total acid formation; (*b*) a restriction in the quantity of sugar used to about 30 % of that normally metabolized; (*c*) a decrease in the yield of citric acid expressed as a percentage of the sugar consumed; and (*d*) an increase in the yield of gluconic acid expressed as a percentage of the sugar consumed.

2. Iodoacetic acid (*M*/1000 or *M*/500 as Na salt) in 10 % sucrose solution in contact with the fully developed mycelium of *A. niger* (strain T1) at 30° caused in a given time: (*a*) a diminished acid formation; (*b*) a large increase in the weight of citric acid formed; and (*c*) a large decrease in the weight of gluconic acid formed, as compared with a control experiment made in absence of iodoacetate.

3. NaF when added at concentrations from *M*/150 to *M*/400 to cultures of *A. niger* on cane sugar depressed the consumption of the sugar, the development of titratable acidity and the formation of citric acid. Spores of *A. niger* did not germinate in a nutrient salt-sugar medium containing *M*/100 NaF, and the same concentration of this poison was found to suppress completely the formation of citric acid by mycelia which had been grown normally before being brought into contact with fluoride.

4. Na iodoacetate (*M*/500) did not prevent the formation of malic and citric acids in cultures of *Penicillium citrogenum* and *P. Johannioli* on a Na acetate solution.

5. Na iodoacetate, $M/500$ or $M/450$, did not prevent the formation of *l*-malic acid from succinic acid by *A. niger* (strain N 1).

6. NaF ($M/150$) did not prevent the formation of *l*-malic acid from succinic acid by *A. niger* (strain N 1).

The results summarized under headings 4, 5 and 6, when considered in conjunction with the other results described in this communication and in Part II [Johnson *et al.* 1937], provide no evidence against the view that succinic and acetic acids are intermediates in the mycological conversion of sugar into citric acid.

REFERENCES

Bernhauer (1928). *Hoppe-Seyl. Z.* **177**, 99, 279.
—— Böckl & Siebenäuger (1932). *Biochem. Z.* **253**, 37.
—— —— —— (1934). *Ergebn. Enzymforsch.* **3**, 209–14.
Butkewitsch (1924). *Biochem. Z.* **154**, 180.
—— & Gajevskaya (1935). *C.R. Acad. Sci. U.R.S.S.* **3** (**8**), no. 9 (**69**), 405.
Chrząszcz & Tiukow (1930). *Biochem. Z.* **229**, 343.
—— —— & Zakormorny (1932). *Biochem. Z.* **250**, 254.
Clutterbuck (1936). *J. Soc. Chem. Ind., Lond.*, **55** T, 67.
Fischer & Passmore (1889). *Ber. dtsch. chem. Ges.* **22**, 2730.
Gudlet (1936). *Ann. Rev. Biochem.* **5**, 604.
Johnson, (1934). Thesis. Manchester University.
—— Knight & Walker (1937). *Biochem. J.* **31**, 903.
Kluyver (1935). *Ergebn. Enzymforsch.* **4**, 251.
Quastel & Whetham (1924). *Biochem. J.* **18**, 519.
Raistrick & Clark (1919). *Biochem. J.* **13**, 329.
Scheffer (1928). Diss. Delft.
Virtanen (1925). *Comment. phys.-Math., Helsingf.*, **2**, 20.
—— & Pulkki (1930). *Ann. Acad. Sci. Fenn.* **33**, no. 3, 1.
—— & Simola (1927). *Hoppe-Seyl. Z.* **163**, 284.
Walker (1936). *J. Soc. Chem. Ind., Lond.*, **55** T, 61.
Wells, Moyer & May (1936). *J. Amer. chem. Soc.* **58**, 555.
Williamson (1918). *J. Amer. chem. Soc.* **40**, 693.

XLVI. THE ACTION OF THE DIPROPIONATE AND BENZOATE-BUTYRATE OF OESTRADIOL ON OVARIECTOMIZED RATS

By V. KORENCHEVSKY, R. BURBANK and K. HALL

From the Lister Institute, London

(*Received 27 January 1939*)

Miescher *et al.* [1938] have prepared several aliphatic di-esters of oestradiol which they have carefully tested, using the methods of vaginal smear and uterine growth. They gave subcutaneous injections of the di-esters to immature normal and ovariectomized rats, the total dose being usually 50 μg., given in equal parts on two consecutive days, and found that, as compared with oestradiol, the di-esters have an extremely intense and prolonged effect. The di-esters are therefore important both for experimental and clinical purposes.

In the present paper are given the results of ten experiments (Tables I and II, groups III–VIII, XII–XV), in which the esters were injected for a period of 3 weeks, and of one experiment (Table I, group IX) for 19 weeks. The dipropionate and the 3-benzoate-17-*n*-butyrate were chosen as being typical representatives of the series of aliphatic and mixed aliphatic-aromatic di-esters.

Two esters, the effects of which last for very different lengths of time, were deliberately chosen: a long duration of effect would appear to be most advantageous for clinical use, but it is necessary to consider also the disadvantage of being unable to stop the effects of the injection if this should be required. In this respect there might even be danger, with a hormone which produces not only physiological but also pathological effects, as is the case with all oestrogens. According to Miescher *et al.* (p. 731, fig. 7) the maximal growth of the uterus with the dipropionate was obtained on the 12th day, after which period the effect started to decline, while with the benzoate-butyrate the maximal growth was still present and had not begun to decline on the 50th day. Robson *et al.* [1938], injecting a total dose of 50 μg. of the benzoate-butyrate into mice, found that the effect of the hormone lasted more than 120 days, but that after 95 days the degree of its action was already halved.

Technique

The hormones were supplied by Messrs Ciba Ltd. Our technique of preparation was as usual: the hormones were first dissolved in alcohol, then mixed with warmed sesame oil and the alcohol distilled off *in vacuo* at a temperature rising slowly from 70 to 85°. The hormonal solution was then put into ampoules, sealed and sterilized for 3 days at 100°. The ampoules were kept in the cold store until needed. While oestradiol benzoate-butyrate was not affected by this method of solution, the dipropionate appeared to be weakened, since after keeping for a few months, even in the cold store, its effect was less strong. Dr Miescher has suggested (personal communication) that with our method of solution some of the ester might possibly be decomposed with liberation of pure oestradiol, and that shaking the dipropionate in oil warmed up to 70° without any additions

gives the most stable solution. However, since we discarded all experiments in which we noticed a weaker effect of the dipropionate and used fresh solutions, the duration of the effect in our experiments has been similar to that in the experiments of Miescher *et al.*

The rats used were ovariectomized on the 22–26th day of age. The hormones were injected as follows: the dipropionate was injected for three consecutive weeks in weekly doses of 18 and 90 μg. (6 and 30 μg. respectively three times a week, Table II, groups XII–XV); the smallest dose 18 μg. of the benzoate-butyrate (Table I, groups III–V) was injected similarly, and the dose of 90 μg. (groups VI–VIII) was given once a week for three consecutive weeks; in group IX, the weekly dose of 300 μg. was given (100 μg. three times a week) for 19 weeks.

In order to ascertain the duration of the effect after the last injection the rats in groups IV, V, VII, VIII, XIII and XV ("10–106 days rest") were left without injection for the requisite number of days before killing. In order that in each experiment all the rats might be killed on the same day, the injections into the rats of groups "10–106 days rest" were started the respective number of days earlier than the injections into the rats which were killed on the next day after the last injection (groups III, VI, IX, XII and XIV).

In this way the final ages of comparable groups were the same. We have not noticed any definite effect through starting the injections at an earlier age in some groups than in others.

Other details of our technique were the same as in our previous experiments. The total number of rats used was 99. The average age of the rats in Table I was 107–176 days (col. X), and in Table II 88 days.

Table I. *Effects of oestradiol benzoate-butyrate on actual weights of organs of ovariectomized rats*

	Control rats		Weekly doses of oestradiol benzoate-butyrate							
			Injected for 3 weeks						Injected	
Organs	Normal I	Ovariec-tomized II	18 μg. III	18 μg. + 30 days rest IV	18 μg. + 52 days rest V	90 μg. VI	90 μg. + 30 days rest VII	90 μg. + 106 days rest VIII	for 19 weeks 300 μg. IX	Average final age in days X
Uterus (horns	411	36	228	276	271	248	290	—	—	107
+cervix, mg.)	549	43	—	—	—	—	—	292	536	176
Vagina with cli-	232	175	314	311	353	258	328	—	—	107
toris (mg.)	326	189	—	—	—	—	—	365	409	176
Preputial glands (mg.)	97	58	73	67	67	78	81	76	58	107–76
Hypophysis (mg.)	14·7	12·5	18·8	23·2	20·2	30·7	42·2	32·0	76·0	107–76
Thymus (mg.)	283	440	397	385	313	257	277	—	—	107
	213	221	—	—	—	—	—	197	110	176
Liver (g.)	8·34	9·08	8·69	11·02	10·67	9·36	9·41	8·22	8·45	107–76
Kidneys (g.)	1·53	1·61	1·82	2·02	1·83	1·99	1·86	—	—	107
	1·87	1·79	—	—	—	—	—	1·92	1·82	176
Abdominal fat (g.)	21	20	10	15	17	12	12	16	7	107–76
Final body wt. (g.)	244	308	245	288	283	247	224	—	—	107
	280	322	—	—	—	—	—	266	198	176
No. of rats in the group	10	9	4	6	3	7	7	4	4	107–76

Note. In the control groups I and II two average weights are given only for those organs of which the weight varies considerably according to the age (column X). In group I the vaginal weight of rats aged 107 days is given without clitoris.

Table II. *Effects of oestradiol dipropionate on actual weights of organs of ovariectomized rats*

Organs	Control spayed rats XI	Weekly dose of oestradiol dipropionate: 18 μg. XII	18 μg. + 10 days rest XIII	90 μg. XIV	90 μg. + 10 days rest XV
Uterus (horns + cervix, mg.)	38	249	210	252	232
Vagina with clitoris (mg.)	170	285	313	268	281
Preputial glands (mg.)	49	74	63	67	65
Hypophysis (mg.)	10·9	22·1	21·3	35·5	32·4
Thymus (mg.)	467	299	313	218	223
Liver (g.)	8·56	9·00	11·35	9·38	9·32
Kidneys (g.)	1·57	1·84	1·97	2·10	1·82
Abdominal fat (g.)	17	9	13	7	11
Final body weight (g.)	276	223	273	223	211
Gain in body weight (g.)	76	21	39	28	27
No. of rats in the group	10	12	8	7	8

Effect on sex organs

The maximum effect, as studied by weight of the organs, was slightly greater with the benzoate-butyrate than with the dipropionate. We have not studied the duration of the effect of the dipropionate for a longer period than 10 days after the cessation of the injections. At the end of this period the weight of the uterus was only slightly decreased, while that of the vagina was slightly increased (groups XIII and XV) as compared with the weights of these organs in rats killed on the day after the last injection (groups XII and XIV). With the benzoate-butyrate, the maximum effect was still fully maintained on the 106th day after the last injection (group VIII).

With both hormones, the normal weight and size of the *uterus* was not obtained with any of the doses. Even with 300 μg. (group IX) the large weight of the uterus was due to accumulation of secretion in the uterine lumen greatly distending the uterus, a similar condition to that observed during oestrus. Such a secretion greatly increases the weight of the uterus, while the control animals in group I were always killed during dioestrus, when this secretion is absent.

Furthermore, with all doses, metaplasia into squamous epithelium was observed in the uterine epithelium (the first stage of "oestrogenic precancerous" changes). This metaplasia, sometimes in a severe form, was still present in some rats even 106 days after the injections were ceased (group VIII). This means that not only physiological recovery but also the pathological effect was maintained and can be present as much as 15 weeks after the last injection.

One of the rats injected with 300 μg. of the benzoate-butyrate developed pyometra in the greatly enlarged uterus (weight 4·3 g.), with adhesions to neighbouring organs. Such an effect has been found previously by other workers after injection of large doses of oestrogens.

In contrast to the uterus, the *vagina* returned to normal or in some groups (VIII and IX) was even larger and better developed than in normal control rats. In the rats killed on the next day or up to about two months after the last injection the vagina had a structure more or less typical of oestrus (cornified epithelial layer).

Progestational changes in the vagina. The most remarkable effect, however, occurred in the group of 4 rats killed 106 days (group VIII) after discontinuance of the injections: in two of these rats the appearance of the vagina was similar

to that found at metoestrus (with some cornification), but in the remaining two the epithelial layer was mucified—in one rat to a degree not far from that found during pregnancy.

From these results the following conclusions may be suggested, but confirmation on a larger number of ovariectomized animals is necessary:

(1) Oestrogenic compounds with a long period of action after discontinuing the injections (e.g. oestradiol benzoate-butyrate) may, towards the end of this period, produce a progestational instead of oestrous effect on the vagina.

(2) Since progesterone is naturally present in gonadectomized animals (e.g. in the adrenals [Beall & Reichstein, 1938; Beall, 1938]), the injected oestrogens, if present in the correct ratio with progesterone, might produce this progestational effect on the vagina.

(3) Probably, in the case of oestradiol benzoate-butyrate this ratio is reached toward the end of the after-period of "106 days rest".

(4) This explanation seems to us more probable than that oestrogens alone, without co-operation with progesterone, when injected in small amounts produce mucification, and when in larger amounts produce cornification of the vagina.

The latter theory, however, has been suggested by the workers who first noted the mucifying effect of very small amounts of oestrogenic concentrates or of purified oestrone [Robson, 1931; Robson & Wiesner, 1931–32; Meyer & Allen, 1932]. The latter authors also review the literature on the subject.

The *preputial glands* in most rats were slightly enlarged but did not return to normal.

Effect on body weight, fat deposition and non-sexual organs

In our previous experiments pure oestrone was injected in doses of 2–18 μg. daily for 4 weeks [Korenchevsky *et al.* 1935] or 6 μg. three times a week for 3 weeks [Korenchevsky & Dennison, 1936; Korenchevsky *et al.* 1936; 1937, 1 and 2] and the rats were killed on the day following the last injection. These doses of this compound did not produce constant changes in the non-sexual organs of ovariectomized females, with one exception—the thymus. The physiological involution of this organ, delayed in ovariectomized rats, was increased by oestrone. In some cases the weight of the liver was slightly decreased and that of the hypophysis slightly increased. A definite decrease in the gain in body weight and fat deposition was noted. Stunted growth after injections of oestrogens has been reported by several authors.

In the present experiments some of the effects observed were similar to those obtained previously, but other changes also occurred.

Body weight and fat deposition. As in our previous experiments, the di-esters produced stunted growth and decreased fat deposition in all groups—the larger the dose, the greater the effect. However, although during the injections the body weight remained stationary or increased less than in the controls, or even in some cases began to fall slightly, in the after-periods of "rest" the body weight and fat deposition began to return towards normal in spite of the persistence of the sex effect. With 18 μg. these restorative changes were much more marked than with 90 μg.

Non-sexual organs. The effect on the *thymus* (increased involution) was the same as in our previous experiments, but the enlargement of the *hypophysis* became more constant and much more pronounced. The larger the dose, the greater was the hypophyseal hyperplasia. With the benzoate-butyrate the hyperplasia increased during the first month after the end of the treatment

(groups IV and VII), but later restorative changes began to develop (groups V and VIII), while the sex effect was still fully maintained. The changes with the dipropionate (groups XIII and XV) were too small to suggest a definite decrease of hypophyseal hyperplasia 10 days after the end of the treatment.

A definite change in the actual weight of the *liver* was not always found during the period of injections, but in the after-periods of "rest" a considerable enlargement occurred (both in actual weight and that per unit of body weight) when small doses were given (groups IV, V and XIII). When the dose was increased up to 90 μg. there was no significant change in the weight of the liver after the injections ceased (groups VII, XV). However, when the period of rest is longer, as the experiment with benzoate-butyrate shows (groups V and VIII), the enlargement of the liver decreases or disappears, in spite of the maintenance of the sex effect.

That the changes in the liver are significant is supported by the fact that, per unit of body weight, the weight of the liver was also increased in all the groups even during the periods of injections. It is clear, therefore, that during the period of the injections, or when large doses of the esters were injected the effect of oestrogens on the liver was veiled by the stunted growth of the animal, but the weights per unit of body weight and the enlargement of the organ after the end of treatment suggest a stimulating action of oestrogens on this organ, provided that histological investigation will confirm this conclusion.

The *kidneys* were enlarged in varying degree in all groups both in actual weight (except group IX) and those per unit of body weight.

In *adrenals and heart* no constant or pronounced changes were observed in the actual weights, but per unit of body weight there was an increase in most of the injected rats.

Thyroids and spleen showed no definite changes in weight.

Comparison of the effects of female hormones on females with those of male hormones on males

If the general results of previous experiments (of about 3 weeks' duration) performed by Korenchevsky and co-workers on castrated male rats injected with male hormones are compared with the present observations, some similarities and differences in the action of male and female hormones on males and females respectively can be noted. Thus, as judged by weights, the di-esters of oestradiol and the male hormones produced similar effects on sex organs, thymus, liver (with the reservations for oestrogens already mentioned) and kidneys. The two groups of hormones, however, in most cases influenced differently the body-weight, fat deposition, hypophysis and adrenals.

Thus the oestrogens do not act on females in the same way as the male hormones on males, although some similar properties are present in both groups.

Summary

1. The effects of the dipropionate and benzoate-butyrate of oestradiol were studied on 99 ovariectomized rats.

2. With the doses used, the effects on the sex organs were maintained in the case of the dipropionate during the investigated period of 10 days without injections, while the action of the benzoate-butyrate had not begun to decline even on the 106th day after the last injection.

3. Complete restoration to normal weight and structure of the vagina but not of the uterus was obtained. Moreover, an important pathological change, squamous metaplasia, occurred in the uterine epithelium and was still present up to 106 days after cessation of the injections.

4. While the restorative effect on the sex organs in the case of oestradiol benzoate-butyrate was maintained at its maximum for more than 106 days, the effect on the structure of the vagina in this later stage of the period without injections may be changed from oestrous to progestational form (mucification of the vaginal epithelium).

5. Most probably this progestational effect on the vagina was due to co-operative action at this period of a small amount of oestrogenic hormone (liberated from the injected depot) with progesterone which has probably originated from the adrenals.

6. Body growth and fat deposition were depressed by the di-esters but a partial recovery took place in the period after the injections.

7. Involution of the thymus was hastened; hyperplasia of the hypophysis was present during and after the period of the injections; pronounced enlargement of the liver during some after-periods, and of the kidneys during and after the periods of injections was noted.

Grants from the Medical Research Council and the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish to express our gratitude to Messrs Ciba Ltd., in particular to Dr K. Miescher, for supplying the hormones.

REFERENCES

Beall (1938). *Biochem. J.* **32**, 1957.
—— & Reichstein (1938). *Nature, Lond.*, **142**, 479.
Korenchevsky & Dennison (1936). *Biochem. J.* **30**, 1514.
—— —— & Brovsin (1936). *Biochem. J.* **30**, 558.
—— —— & Eldridge (1937, 1, 2). *Biochem. J.* **31**, 467 and 475.
—— —— & Levy Simpson (1935). *Biochem. J.* **29**, 2534.
Meyer & Allen (1932). *Science*, **75**, 111.
Miescher, Scholz & Tschopp (1938). *Biochem. J.* **32**, 725.
Robson (1931). *J. Physiol.* **71**, 3 *P*.
—— & Wiesner (1931–32). *Quart. J. exp. Physiol.* **21**, 217.

XLVII. THE MANIFOLD EFFECTS OF PROLONGED ADMINISTRATION OF SEX HORMONES TO FEMALE RATS

By V. KORENCHEVSKY, K. HALL and R. BURBANK

From the Lister Institute, London

(*Received 27 January 1939*)

In his excellent book Simonnet [1937] has collected and analysed the extensive literature on the effects of oestrogenic hormones, while the papers on the effects on females of male hormones and their co-operative and antagonistic activities with oestrogens have recently been reviewed by Korenchevsky [1939].

Several workers have studied the effects of prolonged administration of oestrogenic hormones, but long duration experiments with male hormones on females are urgently needed.

In our previous papers [see references, Korenchevsky, 1937] we have studied the effects of male sex hormones on normal and spayed rats for the comparatively short period of 21 days. It seemed probable that certain weak and inconstant changes, produced especially in the non-sexual organs, might be more clearly defined if the period of administration of the hormones were prolonged. In the present experiments, therefore, the rats were injected for the comparatively long period of 3–3½ months, and, as will be shown in this paper, several aspects of the problem were made clear. Such an investigation is also of clinical importance, since the hormones are often applied for long periods and in large doses in order to obtain the desired results.

In the present experiments the following sex hormones have been investigated:

(1) The natural male hormones, i.e. those which are known to occur naturally in the female organism, namely androsterone [Callow & Callow, 1938] and *trans*dehydroandrosterone [Callow, 1936]. As the "female" activity of these hormones is weak [Korenchevsky *et al.* 1935; Korenchevsky & Dennison 1936, 1, 2], large doses of 7·5 mg. a week were given.

(2) Testosterone propionate was included as possessing the strongest female activity of all the known male hormones [Korenchevsky, 1937; Korenchevsky & Hall, 1937]. Medium (2·25 mg. a week) and large (7·5 mg. a week) doses were used.

(3) Of the oestrogens, oestradiol dipropionate was chosen because according to Miescher *et al.* [1938] it appears to be one of the most interesting esters of oestradiol, with a not too prolonged activity on oestrus and growth of the uterus, but of which otherwise little is known. Hormones with very prolonged activity (e.g. oestradiol benzoate-butyrate) have the disadvantage that, once they have been injected, their effects cannot be checked even if desired, and control over their physiological and pathological activities is lost for a very long period [Korenchevsky *et al.* 1939, 1].

Doses of 18, 90 and 200 μg. a week of oestradiol dipropionate were used alone or simultaneously with the male hormones, in order to investigate their combined effects.

(4) In one group of our experiments, progesterone was given simultaneously with testosterone propionate and large doses of oestradiol dipropionate, the main object of this being to study the property of progesterone of preventing the pathological changes produced by oestrogens in the sexual [Korenchevsky & Hall, 1938, 1] and perhaps in some other organs.

Details of the results of histological investigation will be published elsewhere, a few statements only being included in this paper. Some preliminary results have already been published [Korenchevsky & Hall, 1938, 2].

Technique

The general arrangement of the experiments is clear from Tables I and II. A total of 88 rats was used: 9 normal and 13 spayed animals were kept as controls, and 4 normal and 62 spayed animals were injected with the hormones. The rats in Table I were injected for an average of 87 days, their average age when killed being 131 days. In Table II the average age of the spayed rats in groups XI–XVI was 150 days and that of the normal rats in groups X, XVII and XVIII was 201 days, all the rats having been injected for an average of 109 days. Ovariectomy was performed at the age of 21–24 days.

The male hormones and oestradiol dipropionate were supplied by Messrs Ciba Ltd., progesterone by Messrs Organon Ltd. The hormones were dissolved in sesame oil and were injected: androsterone, *trans*dehydroandrosterone and testosterone propionate five times a week, oestradiol dipropionate three times a week (except the dose of 200 μg. given once a week), progesterone six times a week. The weekly doses are given in the tables. Pure sesame oil was injected into the control rats.

For economy of space the data per unit of body weight have not been tabulated, but will where necessary be referred to in the text.

The other details of experimental technique were the same as in our previous papers.

Table I. *Effects of androsterone and* trans*dehydroandrosterone injected alone or simultaneously with oestradiol dipropionate on actual weights of organs of spayed female rats*

	Controls injected with oil		Spayed rats injected weekly with: Oestradiol dipropionate		Androsterone, 7·5 mg.			Dehydroandrosterone, 7·5 mg.	
	Normal rats	Spayed rats	18 μg.	90 μg.	Alone	+ Oestradiol dipropionate 18 μg.	+ Oestradiol dipropionate 90 μg.	Alone	+ Oestradiol dipropionate 90 μg.
	I	II	III	IV	V	VI	VII	VIII	IX
Uterus (mg.)	448	34	266	283	59	327	357	63	366
Vagina with clitoris (mg.)	292	190	332	350	312	416	440	253	366
Preputial glands (mg.)	92	62	74	54	205	126	104	132	84
Adrenals (mg.)	78	70	85	62	57	73	84	57	63
Hypophysis (mg.)	15·4	12·3	37·3	98·0	10·8	30·3	48·0	11·8	55·5
Thymus (mg.)	261	380	253	178	274	223	125	344	172
Liver (g.)	8·66	8·93	8·17	7·61	11·39	10·01	8·96	9·81	8·90
Kidneys (g.)	1·71	1·61	1·78	1·56	2·25	2·11	2·01	1·64	1·70
Spleen (mg.)	515	618	465	446	739	481	630	824	376
Heart (mg.)	730	769	685	623	928	759	723	768	644
Abdominal fat (g.)	18	20	11	9	20	13	9	19	12
Final body wt. (g.)	230	308	218	205	339	257	228	308	225
Gain in body weight (g.)	126	169	82	73	199	103	112	165	76
No. of rats in group	3	8	3	5	4	4	3	4	4

Table II. *Effects of testosterone propionate injected alone or simultaneously with oestradiol dipropionate and progesterone on actual weights of organs of female rats*

	Controls injected with oil		Spayed rats injected weekly with					Normal rats injected weekly with testosterone propionate	
Organs	Normal rats	Spayed rats	Oestradiol dipropionate 0·2 mg.	Testosterone propionate 2·25 mg.	Testosterone propionate 7·5 mg.	Testosterone propionate 2·25 mg. + oestradiol dipropionate 0·2 mg.	Testosterone propionate 2·25 mg. + progesterone 4·5 mg. + oestradiol dipropionate 0·2 mg.	0·75 mg.	7·5 mg.
	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
Uterus (mg.)	526	33	326	262	395	511	337	383	3295
Vagina with clitoris (mg.)	324	175	311	471	534	484	463	522	611
Preputial glands (mg.)	85	49	50	198	196	203	121	141	245
Adrenals (mg.)	66	63	53	50	62	64	60	47	66
Hypophysis (mg.)	17·6	11·5	132·3	10·6	10·9	60·2	47·2	11·9	11·6
Thymus (mg.)	155	327	128	123	26	75	55	129	59
Liver (g.)	8·78	8·69	6·77	10·42	11·18	7·91	7·65	9·58	9·10
Kidneys (g.)	1·74	1·54	1·52	2·08	2·54	1·86	1·88	1·84	2·51
Spleen (mg.)	642	503	340	913	820	429	387	841	716
Heart (mg.)	816	704	596	935	899	621	632	963	1065
Abdominal fat (g.)	19	22	6	18	12	8	8	17	9
Final body wt. (g.)	273	323	192	314	290	203	221	303	273
Gain in body wt. (g.)	67	161	13	139	73	64	56	92	68
No. of rats in group	6	5	9	7	4	6	9	2	2

Ovariectomized rats

Effect of ovariectomy. In this paper the changes observed after ovariectomy will be only briefly summarized, the conclusions being drawn from the results of our previous and present experiments on rats. The only really striking changes, following ovariectomy performed before sexual maturity, occur in the sex organs, which become atrophic. Of the non-sexual organs, a definite change occurs in the thymus, the physiological involution of which becomes delayed, i.e. the gland is larger than in control rats of the same age. The gain in body weight increases after ovariectomy, this increase being only partly due to the slight increase in the amount of body fat. Unlike castration, ovariectomy did not produce any pronounced or constant changes in the actual weights of adrenals, hypophysis, liver, kidneys, spleen and heart. The weights of these organs when calculated per unit of body weight, however, were in most cases slightly decreased, which is to be explained by the greater body weight of the spayed rats as compared with that of the normal intact animals.

In males, on the other hand, the gain in body weight decreases after castration. Therefore the effects of gonadectomy on the weights of the organs calculated per unit of body weight are more similar in both sexes with liver, kidneys, spleen and heart than they appear from the actual weights, but with hypophysis and adrenals the differences are more pronounced.

Effect of the hormones on sex organs

Oestradiol dipropionate (Table I, groups III and IV; Table II, group XII). With all doses the weight of the vagina reached normal or even supernormal level, but complete restoration of the uterus was not obtained. The largest

uterus was produced by the largest dose of 200 μg. (group XII). No constant changes were found in the preputial glands.

In most cases the vagina showed oestrous cornification of the greatly enlarged epithelial layer, or in some cases swollen epithelial cells with dropsical vacuolation. The uterus was of abnormal fibrotic structure. With the smaller doses (18 and 90 μg.) the epithelial cells were high columnar and in only one rat out of 8 contained a few very small patches of cells with squamous metaplasia (first stage of "precancerous" changes produced by oestrogens). With 200 μg. the metaplasia of the uterine epithelium was present in about 44 % of rats, in some cases being very pronounced and involving the whole layer, with cornification of the upper row of cells.

Androsterone and trans*dehydroandrosterone* (Table I, groups V and VIII). Both these hormones had only a very weak effect on the uterus, not significantly greater than that produced in our previous experiments of only 21–27 days' duration [Korenchevsky *et al.* 1935; Korenchevsky & Dennison, 1936, 1]. A much greater effect, especially with androsterone, was obtained on the vagina and preputial glands. Dehydroandrosterone, as in our previous experiments [Korenchevsky & Hall, 1937], caused mucification of the vaginal epithelium.

The addition of oestradiol dipropionate had a definite co-operative effect (as judged by weights of the organs) on the activity of androsterone, producing even superdevelopment of the vagina (Table I, groups VI and VII), while the co-operative effect with dehydroandrosterone was much less definite, especially on the vagina (group IX). After these simultaneous injections the effects of the oestradiol predominated on the structure of both vagina and uterus: the vagina in most cases showed oestrous changes, while fibrotic and other changes typical for oestradiol were seen in the uterus.

It is very remarkable that the *metaplastic changes in the uterine epithelium* occurred more frequently and were generally of more pronounced degree when androsterone, and apparently also dehydroandrosterone, were injected simultaneously with oestradiol dipropionate, than when the latter hormone was injected alone. With simultaneous injections the metaplastic changes were present respectively in 83 % (groups VI and VII) and 50 % (group IX), as compared with 13 % in groups III and IV (oestradiol alone).

However, before making a definite statement that androsterone and *trans*-dehydroandrosterone increase the "oestrogenic precancerous" changes, it will be necessary to repeat the experiment on a greater number of animals.

Testosterone propionate. When this hormone was injected alone (Table II, groups XIII and XIV), a large uterus and supernormal development of the vagina and preputial glands were obtained.

The structure of both the vagina and uterus was similar to that previously described in experiments of 21 days' duration [Korenchevsky & Hall, 1937], the chief features being: in the vagina—mucification of the epithelium; in the uterus—an increased amount of fibrous tissue in the stroma, hypertrophied muscle cells, and (with the large dose) progestation-like lace foldings of the mucosa, which were, however, much more pronounced and more closely reminiscent of those found during pregnancy than those obtained in our previous experiments of short duration.

The addition of oestradiol dipropionate (group XV) was followed by better growth of the uterus, but severe squamous metaplasia of the uterine epithelium with cornification was present in half of the rats. Testosterone propionate in the dose used (2·25 mg. a week) was unable to prevent these pathological effects of oestrogens. The effect of oestradiol also overruled that of the male hormone in

the vagina, in which the changes were of the oestrous type, though in some cases irregular.

When, however, in another experiment (which will be described in detail elsewhere) a large dose of 7·5 mg. a week of testosterone propionate and a smaller dose (90 μg. a week) of oestradiol benzoate-butyrate were injected, the development of the metaplastic changes was completely prevented, while in the uterus and vagina the effect of testosterone propionate (abortive progestational changes) was present in most cases.

The addition of progesterone to the combined injections of testosterone propionate and oestradiol dipropionate (group XVI) had a definite preventive action on these pathological changes: metaplasia was present only in 1 rat out of 9, and was of a very weak degree. We have shown previously [Korenchevsky & Hall, 1938, 1] that progesterone was able to prevent these metaplastic changes in the uterine epithelium produced by pure oestradiol, and have given the literature relating to the subject.

The special pictures of the uterus with abortive epithelial foldings and of the vagina with vacuolated, swollen epithelium in rats of group XVI will be described elsewhere.

It is also noteworthy that the addition of progesterone was followed by the development of a smaller uterus (group XVI) as compared with that obtained in group XV.

Significance of varying amount of body fat in calculations per unit of body weight

The amount of body fat in the experimental animals varied considerably under the influence of the injections. Therefore there is a possibility that misleading conclusions might be drawn, if the weights of the non-sexual organs calculated per unit of body weight of fat rats (e.g. control groups, especially spayed rats) are compared with those of thin animals (e.g. those injected with oestradiol). The depots of inert adipose tissue require less activity from the organs regulating metabolism than do active tissues such as glandular, muscular etc. Consequently different requirements might influence differently the size and weight of the "metabolic" organs.

When studying the non-sexual organs, therefore, we have calculated the weights not only per unit of final body weight, but also per unit of "fat-free" body weight. This latter weight was obtained approximately by subtracting the total body fat from the final body weight (given in the tables) assuming that the abdominal fat (also given in the tables) represents about 30–32 % of the total fat in the body (for details see our previous paper [Korenchevsky *et al.* 1939, 2]. These calculations, however, gave results differing only in degree from the usual calculations per unit of body weight, without changing the essence of the conclusions.

Effect on gain in body weight and fat deposition

Growth, as judged by gain in body weight, was slightly stimulated by androsterone (group V), little or not at all affected by dehydroandrosterone and small doses of testosterone propionate (groups VIII and XIII) and depressed by oestradiol dipropionate (considerably, groups III, IV and, especially, XII) and large doses of testosterone propionate (slightly, group XIV).

The amount of body fat was considerably decreased by oestradiol dipropionate (the larger the dose the greater the decrease) and to a less degree by large doses of testosterone propionate, but unchanged by the other hormones or doses.

The remarkable neutralizing effect of the male hormones on oestrogens was again demonstrated by their power, when simultaneously injected, of preventing in most cases, and sometimes to a considerable extent, the depressing effect of the female hormone on body weight and fat deposition (groups VI, VII, IX, XV, XVI).

Effect of the hormones on non-sexual organs

Adrenals. The actual weights of these glands were slightly changed by oestradiol dipropionate, the smallest dose causing an increase (group III), the other doses a decrease (groups IV and XII). When calculated per unit of body weight, the weights showed with all doses a constant increase of 30–70%. This suggests that in spite of the evidence of actual weights, oestradiol dipropionate may have in reality some stimulating action on the adrenals, but fibrous degenerative changes found in the cortex did not support this suggestion.

The male hormones when injected alone (groups V, VIII and XIII, but not the large dose of testosterone propionate, group XIV) in most cases decreased both the actual weights of the adrenals and those per unit of body weight. Thus, in these prolonged experiments the effects of these hormones were similar in females and in males. In experiments of shorter duration on females [Korenchevsky *et al.* 1935; 1936; 1937, 1; Korenchevsky & Dennison, 1936, 1] the effects were similar but with some doses less definite and constant. Testosterone dipropionate also produced smaller adrenals [Korenchevsky *et al.* 1939, 3].

When male and female hormones were injected simultaneously (groups VII, IX, XV and XVI) the activity of the female hormone in most cases changed the effect of the male compounds, the adrenals becoming larger than those of rats injected with the respective male hormones alone. A similar effect was noted with some hormones in our previous experiments (mentioned above) of shorter duration.

Hypophysis. As with other oestrogens, oestradiol dipropionate produced hyperplasia of the hypophysis, the larger the dose the greater being the degree of hyperplasia (groups III, IV and XII). It is remarkable that while male hormones have no definite effect upon the weight and size of the gland (groups V, VIII, XIII and XIV), they can to some extent prevent the hyperplastic effect of oestrogens (groups VI, VII, IX, XV). If in addition progesterone is given (group XVI), the neutralizing effect appears to be even greater; the hypophyses of two rats in this group weighed only 19·8 and 25·6 mg. as compared with an average of 132 mg. with oestradiol alone (group XII). The weight of the hypophysis was thus restored nearly to normal, the average weight in normal controls being 17·6 mg.

Liver, kidneys, heart and spleen. With a few exceptions, the character of the changes in these organs was similar.

Oestradiol dipropionate (groups III, IV and XII) caused a decrease (considerable with large doses) in the actual weights of these organs, except the kidneys, while the weights of all of them per unit of body weight were increased. Therefore, as in the case of the adrenals, the ratio of the weights of these organs to body weight was greater than in control ovariectomized rats and often similar to that in control normal animals.

On this basis the influence of oestradiol on these organs cannot be considered as a true depressing effect, but may even suggest some stimulation, hidden by the effect of the stunted growth of the rats on the weight and size of these organs provided that histological investigation confirms this conclusion. This is also supported by experiments of shorter duration (21 days) with oestradiol dipropionate and oestradiol benzoate-butyrate [Korenchevsky *et al.* 1939, 1], when

the weights, both actual and per unit of body weight, of liver and kidneys were increased by those doses which did not cause much depression of body weight.

On the other hand, the effect of *male hormones* on liver, kidneys, heart and spleen appears to be definitely stimulating, both the actual weights and those per unit of body weight being increased (groups V, VIII, XIII, XIV). Even in experiments of shorter duration, similar results were obtained in some cases [e.g. Korenchevsky *et al.* 1939, 3].

This stimulating effect is further demonstrated by the property of androsterone and testosterone propionate of at least partly preventing the depressing effect of oestradiol on the actual weights of these organs (groups VI, VII, XV). The addition of progesterone (group XVI) does not increase this effect of testosterone propionate.

The weakest stimulant in this respect amongst the male hormones was dehydroandrosterone, which had a definite effect on liver and spleen, but not on heart and kidneys. It also possessed in only a very weak degree the property of preventing the changes produced by oestradiol.

Thyroids. The weights of these organs were unchanged.

Comparison of the effects of the sex hormones on the non-sexual organs in females with those in males

As shown above, gonadectomy does not produce such constant and definite effects on the majority of the non-sexual organs in females as in males. In spite of this, however, the effects of the male and female hormones on adrenals, hypophysis, liver, kidneys, spleen, heart, fat deposition and gain in body weight were similar in both sexes (compare the present results with those obtained in similarly arranged experiments on males [Korenchevsky *et al.* 1939, 2]. The remarkable property of male hormones of more or less neutralizing the effects of the female hormone was also present in both sexes. It should be noted, however, that while in males the weights of liver, kidneys, heart and spleen were restored to normal by male hormones, in the female these hormones produced supernormal development of these organs.

Effect of testosterone propionate on normal rats

Only six rats were used in this preliminary experiment, two in each of the injected groups and two control animals. The results, however, were constant, clear-cut and in many respects in good agreement with those of our previous experiments of shorter duration (21 days) [Korenchevsky *et al.* 1937, 2].

In normal females (groups XVII and XVIII) the effect of testosterone propionate on most of the organs investigated was similar in character to that in ovariectomized rats, but usually differed in degree. The following changes in weight (both actual and per unit of body weight) were found: a "gigantic" uterus with the large dose, and greatly enlarged vagina and preputial glands with both doses; decrease in weight of the adrenals with the small dose and no change with the large dose; decreased weight of hypophysis, and enlargement, varying in degree, of liver, kidneys, spleen and heart. The small dose caused increased gain in body weight but no definite change in fat deposition, while with the large dose fat deposition was decreased but the body weight remained unchanged. As in ovariectomized rats, therefore, the large dose affected fat deposition and body weight more than the small dose.

Some unusual features of the changes in the uterus and vagina must be mentioned. With the small dose there was a decrease in size of the uterus (group XVII), but the structure was normal, with few and low foldings of the

mucosa. The small size of the uterus might be explained by the depressing effect of testosterone propionate on the secretion of gonadotropic hormone from the hypophysis, this dose of the hormone being unable to counterbalance the depressing effect by direct stimulation of the uterus. Analogous results were obtained previously [Korenchevsky & Hall, 1939] in normal male rats after injection of androsterone and *trans*dehydroandrosterone, and the mechanism of this phenomenon was discussed in detail (p. 376). The increase in size of the vagina, however, does not accord with this theory, and therefore at present it is difficult to find a satisfactory explanation of the decrease in size of the uterus, especially on the basis of the results of an experiment on two rats only.

The "gigantic" uterus (group XVIII), produced by the large dose, was distended with secretion, the distension being reminiscent of that during oestrus (the columnar epithelium was normal and the myometrium hypertrophied). The epithelial layer of the vagina was narrow with narrow, mucified cells.

Summary

1. Androsterone, *trans*dehydroandrosterone, testosterone propionate and oestradiol dipropionate, alone, or each of the male hormones in combination with oestradiol dipropionate (in one group also with progesterone) were injected into ovariectomized and normal rats for a period of about 3½ months.

Effects on sex organs of ovariectomized rats

2. With oestradiol dipropionate alone, the vagina was restored to normal or supernormal weight, with normal (usually "oestrus") structure, but not even a weekly dose of 0·2 mg. was able to produce normal size and weight of the uterus, although this dose caused pronounced pathological changes in the structure of this organ.

3. The weak effects of androsterone and *trans*dehydroandrosterone on the uterus were not significantly increased, as compared with experiments of shorter duration, but the vagina and especially the preputial glands were considerably hypertrophied.

4. There was a co-operative effect between oestradiol dipropionate and androsterone or (to a lesser degree) *trans*dehydroandrosterone on the weight and size of the sex organs, but at the same time the number of rats affected with squamous metaplasia of the uterine epithelium (first step of "precancerous" oestrogenic effect) was increased and this condition became more severe.

5. Testosterone propionate produced pronounced progestational changes in the uterus and less strong ones in the vagina.

6. Testosterone propionate had a co-operative effect with oestradiol dipropionate on the weight and size of the uterus, but the occurrence and degree of epithelial squamous metaplasia in this organ were about the same as with the female hormone alone.

7. If, however, progesterone was added to the combination of male and female hormones, or a large dose of testosterone propionate was injected with oestradiol, these "precancerous" changes in the uterine epithelium were prevented to a remarkable degree.

Effects on non-sexual organs

8. Male hormones caused decrease in the weight and size of the adrenals of females similar to that previously described in male rats, while the effect of the female hormone was characterised by peculiar pathological changes. Enlarge-

ment of the adrenals in most cases followed simultaneous injections of male and female hormones.

9. The tumour-like hyperplasia of the hypophysis produced by the oestrogens was neutralized to a considerable extent (in two rats almost to normal) by male hormones.

10. Male hormones caused hypertrophy (in most cases exceeding normal level) of liver, kidneys, spleen and heart, but the female hormone produced either a slight decrease in their actual weights, or (kidneys) no change.

11. Oestradiol dipropionate produced stunted growth and decreased fat deposition, but with the male hormones this depressing effect was absent or (with the large dose of testosterone propionate) slight.

12. When injected simultaneously the male hormone more or less completely neutralized the effect of the oestrogen on liver, kidneys, heart, spleen, fat deposition and body weight.

Effect on normal rats

13. The effects produced by testosterone propionate on the weight and size of most organs of normal rats were in general similar to those in ovariectomized rats, in most cases differing in degree. A remarkable feature was the "gigantic" uterus distended with oestrus-like secretion.

14. Prolonged administration of male sex hormones produced in general similar results in both sexes.

Grants from the Medical Research Council and the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish to express our gratitude to Messrs Ciba Ltd., in particular to Dr K. Miescher, and to Messrs Organon Ltd. for supplying the hormones.

REFERENCES

Callow (1936). *Chem. Ind.* **55**, 1030.
—— & Callow (1938). *Biochem. J.* **32**, 1759.
Korenchevsky (1937). *Brit. med. J.* **2**, 896.
—— (1939). *Ergebn. Vit. und Hormonforsch., Lpz.*, **2**, 418.
—— & Dennison (1936, 1). *Biochem. J.* **30**, 1514.
—— —— (1936, 2). *J. Path. Bact.* **42**, 91.
—— & Hall (1937). *J. Path. Bact.* **45**, 681.
—— —— (1938, 1). *J. Obstet. Gynaec.* **45**, 22.
—— —— (1938, 2). *Nature, Lond.*, **142**, 998.
—— —— (1939). *Brit. med. J.* **1**, 4.
—— Burbank & Hall (1939, 1). *Biochem. J.* **33**, 366.
—— Dennison & Brovsin (1936). *Biochem. J.* **30**, 558.
—— —— & Eldridge (1937, 1). *Biochem. J.* **31**, 475.
—— —— & Hall (1937, 2). *Biochem. J.* **31**, 780, 1434.
—— —— & Levy Simpson (1935). *Biochem. J.* **29**, 2534.
—— Hall & Ross (1939, 2). *Biochem. J.* **33**, 213.
—— —— Burbank & Ross (1939, 3). *Biochem. J.* **33**, 36.
Miescher, Scholz & Tschopp (1938). *Biochem. J.* **32**, 725.
Simonnet (1937). L'hormone folliculaire en physiologie normale et pathologique. Paris.

XLVIII. PRELIMINARY EXTRACTION OF GONADOTROPHIC PRINCIPLE FROM PREGNANT MARE SERUM

By H. RINDERKNECHT, R. L. NOBLE[1] and P. C. WILLIAMS[2]

From the Courtauld Institute of Biochemistry, Middlesex Hospital, W. 1

(*Received 31 January 1939*)

The serum of the mare contains a relatively high concentration of gonadotrophic material during the second and third months of pregnancy [Cole & Hart, 1930]. This substance resembles anterior pituitary extracts in causing development of the follicles, luteinization and interstitial cell proliferation in the ovary of normal and hypophysectomized rats [Evans *et al.* 1933, 1, 2; Rowlands, 1938] while in the hypophysectomized male spermatogenesis is maintained or restored and the interstitial cells are stimulated [Evans *et al.* 1933, 3; Liu & Noble, 1939]. As a result of gonad stimulation secondary effects are produced in both sexes.

In the present paper a simple method is described for separating the active principle from the bulk of inert material in the serum.

Methods

Serum was obtained from the freshly clotted blood of mares at a suitable stage of pregnancy. It was stored at 0° and maintained at as low a temperature as practicable during all procedures.

The gonadotrophic activity was assayed by the increase in weight produced in the ovary of immature (40–50 g.) female rats. Since the supply of serum was limited groups of only 3 rats could be used for each test. Daily injections were made for 5 days and the animals killed on the sixth. The organs were dissected, fixed in Bouin's fluid and weighed from 70% alcohol.

Results

Precipitation with salicylsulphonic acid. An attempt was made to discover a method of removing the inactive material in the serum from the active substance. According to a British patent (specification No. 440,530 (19. ii. 1935)) protein precipitants such as picric acid, salicylsulphonic acid and trichloroacetic acid have been used for this purpose. Preliminary experiments in which picric acid or salicylsulphonic acid was added to the serum to complete precipitation gave inactive filtrates, but by suitably adjusting the concentration, and consequently the *p*H of the salicylsulphonic acid, it was found possible to obtain the required separation. The following standard method was employed for each precipitation, the concentration of acid being altered systematically. To 20 ml. of serum at 0° varying amounts between 1 and 2 ml. of 33·3% salicylsulphonic acid were added with constant stirring. The mixture was kept in the refrigerator for about 10 min., and was then centrifuged in cooled buckets. The clear supernatant fluid was decanted; its *p*H was determined by glass electrode and it was neutralized immediately with 2*N* NaOH. The precipitate was washed with 5 ml. of acid solution. After centrifuging the supernatants were pooled and adjusted to *p*H 7·5 using bromothymol blue as indicator.

[1] Work done during tenure of Leverhulme Research Fellowship, Royal College of Physicians.

[2] Working on a grant from the Medical Research Council.

The results are summarized in Fig. 1, the weights of the ovaries being plotted against the *p*H of the different precipitations. Each test was performed on the equivalent of 2 ml. of the original serum which gave ovaries of 316 mg. when tested. From an examination of the curve it can be seen that the yield of activity

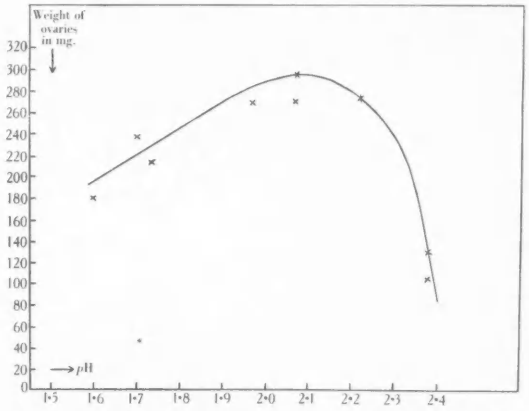


Fig. 1. Relationship between yield of gonadotrophic substance in salicylsulphonic acid supernatant and *p*H.

at *p*H 1·9–2·2 is almost 100%. At *p*H >2·2 the decrease in potency is very rapid. Below *p*H 2·2 it is more gradual and probably due to destruction by the acid rather than to partial precipitation of the active material. It is important to use fresh yellow serum for this method since haemolysed serum requires much more salicylsulphonic acid to obtain the required *p*H and even then there is a considerable loss of potency in the extraction. A similar series of precipitations was carried out with normal horse serum to obtain the quantitative relationship between the *p*H of the precipitation and the weight of total solids in the supernatant. The supernatant solutions after dialysis to remove salicylsulphonic acid and salts were therefore evaporated to dryness and the solid content determined.

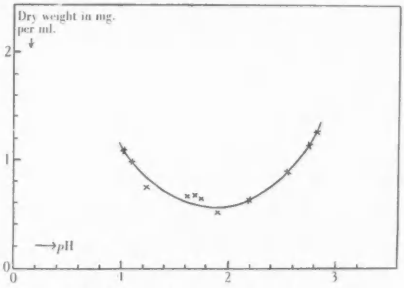


Fig. 2. Relationship between yield of dry substance in salicylsulphonic acid supernatant and *p*H.

From Fig. 2 it may be seen that a minimum yield of solid material is obtained from the supernatant at that *p*H where there is a maximum yield of gonadotrophic hormone, when pregnant mare serum is used.

Further concentration of the gonadotrophic principle was obtained following dialysis of the supernatant in a collodion thimble for 15–20 hr. at 4°. No loss of activity followed this treatment, and the solution after dialysis was evaporated to dryness *in vacuo* at low temperature, or precipitated with 80% acetone. The yield of dry substance after such procedure was 100–150 mg. per 100 ml. of serum. It was found that if the solution after dialysis was concentrated to a small volume a precipitate was formed which could be removed by centrifuging. As this precipitate contained no activity the weight of dry active material could be reduced to 30–50 mg. per 100 c.c. of serum.

Acetone precipitation. A small amount of the active material was obtained by the above process and treated by fractional precipitation with acetone. The active substance was quite soluble in 50% acetone or alcohol, but was precipitated quantitatively in concentrations above 80% acetone. In one experiment a solution containing 6·5 mg. of the dry powder per ml. was adjusted to *p*H 7·5. Following the addition of an equal volume of acetone the precipitate was allowed to flocculate in the cold and was then centrifuged. The precipitate (PMS 8083) on assay was found to contain from 20 to 30% of the total activity. The acetone concentration of the supernatant fluid was raised to 80% and the further precipitate washed with acetone and ether and dried *in vacuo*. This powder (PMS 8082) contained 70 to 80% of the total gonadotrophic activity. This experiment was repeated using a more dilute solution (PMS 83—3 mg. per ml.) since the activity in the 50% acetone precipitate might be due to adsorption. An equal volume of acetone added to this solution only produced a faint opalescence and no definite precipitate was obtained until the *p*H was brought to 5·0. There was no activity in this precipitate but that obtained with 80% acetone contained 100% (PMS 831).

The dry weight of the final products represented 0·13 mg. and 0·09 mg. per ml. of the original serum respectively. Table I gives the activities of these various

Table I

No.	Preparation	Dose per rat: Equiv. to ml. of serum ml.	Dose per rat: Wt. mg.	Ovaries mg.	Uterus mg.
PMS 801	Salicylsulphonic acid supernatant	1	(1)	64	154
PMS 8081	Insoluble part of supernatant	5	—	15	30
PMS 8083	50% acetone precipitate	1	—	26	147
PMS 8082	50% acetone supernatant	1	0·13	44	107
PMS 83	Salicylsulphonic acid supernatant	1	(0·25)	66	267
PMS 831	50% acetone supernatant	1	0·09	67	141

samples, the comparatively low ovary weights being due to the use of haemolysed serum and consequent loss of activity during the salicylsulphonic acid precipitation. The final product is a stable powder readily soluble in water. In concentrations of 3 mg. per ml. it gives neither Millon nor biuret tests. Since these tests are faintly positive when applied to serum proteins in the same concentration, this indicates that the final material is partly non-protein.

Lack of material prevented further research into this acetone fractionation but it appears that the optimal conditions for precipitation are dependent on the *p*H and on the concentration of material.

Under the conditions described it is possible, therefore, to accomplish an approximately 1000-fold concentration (on the basis of total solids) when compared with the crude serum, by a relatively simple and rapid chemical process.

In spite of the marked purification which can be produced, the greater part of the product is inactive material since the above process yields approximately the same weight of dry substance when applied to non-pregnant horse serum.

Discussion

A number of chemical procedures have been described for the purification of the gonadotrophic factor of pregnant mare serum. Goss & Cole [1931] fractionated pregnant mare serum by precipitation with sodium sulphate but only obtained a 10-fold purification. Evans *et al.* [1933, 4] used acetone-ammonia extraction of a solution of the acetone precipitate from the serum. This was applied by Catchpole & Lyons [1934] to fresh serum without any appreciable separation from the serum proteins. Later Evans *et al.* [1933, 5] obtained a purer product by adsorption of the same material on aluminium hydroxide; this procedure was modified and adapted to fresh serum by Gustus *et al.* [1936]. Finally, Evans and his co-workers [1936] claimed to effect a separation of the follicle-stimulating and luteinizing fractions by fractional precipitation with ammonium sulphate. A laborious method of fractional precipitation with acetone or alcohol designed by Cartland & Nelson [1937] yielded a 130- to 1800-fold purification of the active principle. Severinghaus *et al.* [1938] obtained promising results using the ultracentrifuge. The results here described indicate that salicylsulphonic acid precipitation of the inert material is a simple and rapid means of concentration. The further purification following dialysis, and the fractional precipitation with acetone yields the active substance in a form in which it would appear to be well suited for further chemical study.

Summary

A method is described by which a purified fraction of gonadotrophic hormone may be obtained from pregnant mare serum, the essential steps being the precipitation of the inert serum proteins by a suitable concentration of salicylsulphonic acid, and subsequent purification by dialysis and fractional precipitation with acetone. The material obtained was a white powder readily soluble in water, and represented approximately a 1000-fold purification of the hormone in terms of solid content of original serum.

We wish to record our gratitude to Dr A. S. Parkes of the Medical Research Council Staff and Mr F. Day of the Cambridge School of Agriculture for supplies of pregnant mare serum; also to Prof. E. C. Dodds for his interest and criticism.

REFERENCES

Cartland & Nelson (1937). *J. biol. Chem.* **119**, 59.
Catchpole & Lyons (1934). *Amer. J. Anat.* **55**, 167.
Cole & Hart (1930). *Amer. J. Physiol.* **93**, 57.
Evans, Korpi, Simpson & Pencharz (1936). *Univ. Calif. Publ. Anat.* **1**, 275.
—— Meyer & Simpson (1933, 1). *Mem. Univ. Calif.* **11**, 151.
—— —— —— (1933, 4). *Mem. Univ. Calif.* **11**, 67.
—— —— —— & Pencharz (1933, 2). *Mem. Univ. Calif.* **11**, 253.
—— —— —— —— (1933, 3). *Mem. Univ. Calif.* **11**, 301.
—— Gustus & Simpson (1933, 5). *J. exp. Med.* **58**, 569.
Goss & Cole (1931). *Endocrinology*, **15**, 214.
Gustus, Meyer & Woods (1936). *J. biol. Chem.* **114**, 59.
Liu & Noble (1939). In Press.
Rowlands (1938). *J. Physiol.* **92**, 197.
Severinghaus, Levin & Chiles (1938). *Endocrinology*, **23**, 285.

XLIX. SYNTHESIS OF STEROID GLUCURONIDES

By E. SCHAPIRO

From the Pharmaco-therapeutic Laboratory of the University of Amsterdam

(*Received 27 January 1939*)

The steroid glucuronides have acquired fresh interest since Marrian and his co-workers [Cohen & Marrian, 1936; Cohen *et al.* 1936] succeeded in isolating oestriol glucuronide from pregnancy urine, and Venning & Browne [1936] were able in a similar way to recover pregnandiol glucuronide from the urine of pregnant women. According to Marrian's data oestriol glucuronide is about 28 times weaker as an oestrogen than free oestriol. There is evidence that pregnandiol glucuronide is the form in which the corpus luteum hormone, progesterone, is eliminated; the latter cannot be condensed with glucuronic acid unless it is previously reduced to the hydroxylated pregnandiol.

The "male" sex hormones are also eliminated in urine in combined forms, although it is not yet known whether the excreted compounds are also glucuronides.

Glucuronides of steroids have not yet been made synthetically; in the present paper the synthesis of the following is recorded: methyl dehydroandrosterone-triacetylglucuronate; dehydroandrosterone-glucuronic acid; methyl testosterone-triacetylglucuronate; methyl α-oestradiol-3-benzoate-17-triacetylglucuronate; α-oestradiol-17-glucuronic acid; methyl oestrone-triacetylglucuronate; methyl cholesterol-triacetylglucuronate.[1]

The synthetic preparation of glucuronides has become possible, since Goebel & Babers [1935] succeeded in preparing methyl acetobromoglucuronate, which is the necessary starting-point for the synthesis of these substances. When methyl acetobromoglucuronate and the appropriate sterol derivative are shaken in an indifferent medium, such as benzene with silver carbonate, glucuronide formation readily occurs.

The methyl steroid-triacetylglucuronates crystallize well. They are soluble in organic solvents, such as benzene and chloroform, and fairly readily soluble in hot absolute alcohol, from which they may be recrystallized.

The barium salts of the steroid-glucuronic acids can be prepared by hydrolysis of the acetylated methyl esters with baryta solution, and afford the steroid-glucuronic acids on treatment with acids. Dehydroandrosterone-glucuronic acid and α-oestradiol-17-glucuronic acid prepared in this way are characterized by their solubility in dilute alcohol; they are somewhat soluble also in boiling water.

Dehydroandrosterone-glucuronic acid can be recrystallized from absolute alcohol. α-Oestradiol-glucuronic acid crystallizes from very dilute alcohol with 1·5 mol. of water of crystallization, which are removed only on drying in a high vacuum at 115–120°. Also even after repeated recrystallization α-oestradiol-17-glucuronic acid always contains 0·86–1·1 % of ash; further purification of the substance is accompanied by heavy losses of material.

[1] The hormones necessary for this investigation were very kindly placed at my disposal by the N. V. Organon, Oss (Holland). According to a private communication, Dr Luchs, of the Research Department N. V. Organon, Oss, tried to make esters and ethers of oestrone in 1934. This research could not be continued on account of Dr Luchs' illness.

Experimental

I. *Methyl dehydroandrosterone-triacetylglucuronate.* Methyl acetobromoglucuronate (700 mg.) and dehydroandrosterone (500 mg.) were dissolved in benzene (25 ml.) and the solution shaken for 24 hr. with 1 g. of dry Ag_2CO_3. The solution, which was free from bromine, was centrifuged and the residue extracted repeatedly with warm benzene. The combined clear liquors from the centrifuge were evaporated *in vacuo* to a syrup, which crystallized spontaneously on rubbing with a little absolute alcohol. After standing in the ice chest for several hours, the crystals were filtered off, washed with a little cold alcohol and dried. Yield: 255 mg. (24 %), M.P. 193–196°,[1] unchanged after recrystallization from absolute alcohol. (Found: C, 63·88; H, 7·13 %; $C_{32}H_{44}O_{11}$ requires C, 63·54; H, 7·33 %.) The compound had $[\alpha]_D^{20°}$ −19·7° (c=0·0033 in $CHCl_3$) or −16·2° (c=0·0063 in benzene).[2]

220 mg. were dissolved in 20 ml. of hot absolute methyl alcohol and to the hot solution were added 4 ml. of 0·4 *N* $Ba(OH)_2$. A fine, white precipitate was soon formed. The mixture was boiled for 45 min. under a reflux (protected by a soda-lime tube), cooled, treated with 20 ml. of water and kept at 0° for several hours. The crystalline precipitate, consisting of fine needles, was filtered off, washed well with water and dried. Yield: 178 mg. (92 %).

II. *Dehydroandrosterone-glucuronic acid.* 172 mg. of the above Ba salt were digested on the water bath for 30 min. with 1·3 ml. of 0·25 *N* H_2SO_4 and 5 ml. of 50 % methyl alcohol when the coarsely powdered Ba salt of the condensed glucuronic acid was replaced by finely divided $BaSO_4$. The warm suspension was centrifuged and the residue repeatedly extracted with warm 50 % methyl alcohol. The combined centrifugates were evaporated in a vacuum to incipient turbidity. On cooling, the solution soon began to deposit a very finely divided crystalline precipitate, which was filtered off after 24 hr., washed with ice-cold water containing methyl alcohol and dried in a vacuum over phosphorus pentoxide. It formed irregular scales with a mother of pearl lustre. Yield: 114 mg. (76 %).

After recrystallization from absolute alcohol, thick, glittering fragments of prisms were obtained with M.P. 253–254°. Two further recrystallizations from absolute alcohol afforded 49 mg. melting at 254–257°. Finally the substance was recrystallized from 3·5 ml. of 80 % alcohol, when the M.P. rose to 262–264° (constant). The crystals became yellow at about 185–190°, opaque at about 210° and finally melted with decomposition. Yield of pure substance: 25 mg. (Found: C, 64·05; H, 7·79 %. $C_{25}H_{36}O_8$ requires C, 64·62; H, 7·82 %.)

III. *Methyl testosterone-triacetylglucuronic acid.* Methyl acetobromoglucuronate (700 mg.) and testosterone (500 mg.) were dissolved in benzene (25 ml.) and the solution shaken for 24 hr. with 1 g. of dry Ag_2CO_3. The debrominated solution was worked up as described under (I). The syrupy residue was dissolved in a little alcohol and the solution diluted with water. Crystallization occurred after a long time in the ice chest. The crystals were filtered off, washed with cold dilute alcohol and dried. After two recrystallizations from absolute alcohol and one from 96 % alcohol, 26 mg. of a substance were finally obtained which melted at 186–189° and crystallized in beautiful needles.

[1] All melting points were determined in a micro-melting point apparatus according to Kofler.

[2] The value for the rotation, which we have recently given in *Nature, Lond.*, **142**, 1036 (1938), was the result of a single determination and can only be considered as preliminary.

A crystalline mixture was obtained from the mother liquors, from which fractional crystallization from 50% and finally from 70% alcohol afforded a further 84 mg. (about 10·5%) of pure substance.

Methyl testosterone-triacetylglucuronate crystallizes with 1 mol. of alcohol of crystallization, which is lost at 78°, the crystals becoming opaque. (Found: C, 62·49; H, 7·51%. $C_{32}H_{44}O_{11}$, C_2H_5OH requires C, 62·73; H, 7·75%.) The compound had $[\alpha]_D^{20°}+28·3°$ ($c=0·006$ in $CHCl_3$).

IV. *Methyl α-oestradiol-3-benzoate-triacetylglucuronate.* Methyl acetobromoglucuronate (500 mg.) and the equivalent amount of α-oestradiol-3-benzoate were dissolved in benzene (20 ml.), shaken for 24 hr. with 1 g. of dry Ag_2CO_3, and the product worked up as described in (I). The resultant syrup crystallized spontaneously when rubbed with a little absolute alcohol. Yield of crude product: 303 mg.; M.P. 165–175°.

After two recrystallizations from absolute alcohol, about 234 mg. (27%) of pure substance were obtained, crystallizing in fine, felted needles, M.P. 188–191·5°. (Found: C, 65·73; H, 6·40%. $C_{38}H_{44}O_{12}$ requires C, 65·88; H, 6·51%.) $[\alpha]_D^{20°}+9·2°$ ($c=0·0065$ in $CHCl_3$).

223 mg. were dissolved in 30 ml. of methyl alcohol and 5 ml. of 0·33 *N* $Ba(OH)_2$ and 5 ml. of water added to the solution. The Ba salt soon crystallized out in fine needles. The solution was boiled gently for 1 hr. under reflux (soda-lime tube) and, after cooling and diluting with water, the precipitate was filtered off and washed with hot water until the smell of methyl benzoate had disappeared. The salt was then dried in a vacuum over phosphorus pentoxide. Yield: 125 mg. (75%).

V. *α-Oestradiol-17-glucuronic acid.* 125 mg. of the above Ba salt were dissolved in 25 ml. of warm alcohol with the addition of 3·5 ml. of 0·1 *N* HCl and the warm solution was treated drop by drop with 0·1 *N* H_2SO_4 until no further turbidity was produced. In this connexion it was observed that the precipitation of the Ba must take place hot, since in the cold an excess of acid precipitates the unchanged Ba salt. Nevertheless a slight excess of H_2SO_4 is an advantage. The hot liquid was centrifuged and the residue extracted repeatedly with hot dilute alcohol. The combined, clear, acid centrifugates were carefully evaporated in a vacuum, after which crystallization soon occurred in the weakly alcoholic solution in the cold. After a long time in the ice chest, the precipitate was filtered off with suction and washed with ice-cold water containing methyl alcohol. The crystals were extremely thin, long, platelets arranged in rosettes. Yield: 87·5 mg. (80%).

After recrystallizing twice from aqueous methyl alcohol, 56 mg. (about 52%) were obtained, melting at 191–194·5° after drying in a high vacuum at 90° (the crystals sintered at 140–150°). (Found (ash-free): C, 61·02; H, 7·41%; $C_{24}H_{32}O_8$, 1·5 H_2O requires C, 60·60; H, 7·41%.)

The substance was dissolved in alcohol, and the solution treated with 0·1 *N* HCl and a few drops of 0·1 *N* H_2SO_4 in the cold. After 24 hr. in the ice chest the solution was scarcely turbid; it was clarified by filtration and part of the alcohol removed very carefully in a vacuum. Crystallization soon began in the form of small, irregular platelets. The gelatinous nature of the precipitate made filtration extremely slow. The pasty precipitate was again dissolved in a little alcohol and precipitated with water. The operation was repeated until the filtrates were free from acid. The substance was then dried, first in an ordinary vacuum and then in a high vacuum at 115–120°, when a faint tinge of yellow appeared. At this stage the carbon content was found to be too low and the hydrogen content too high; the substance was therefore again dried to constant weight in a high vacuum at

117°, when it lost a further 3·41 % of water and the slight yellowing did not increase. (Found (ash-free): C, 64·19; H, 7·57 %. $C_{24}H_{32}O_8$ requires C, 64·25; H, 7·19 %.)

VI. *Methyl oestrone-triacetylglucuronate.* Oestrone (142 mg.) and methyl acetobromoglucuronate (208 mg.) were dissolved in warm benzene (50 ml.) and the solution shaken overnight with 0·5 g. of dry Ag_2CO_3, after which the mixture was warmed for 30 min. on the water bath. The suspension was then centrifuged and the clear centrifugate worked up as usual. 52 mg. of substance were obtained, which was very sparingly soluble in cold absolute alcohol. After four recrystallizations from absolute alcohol-chloroform the M.P. was 225·5–228°. (Found: C, 63·28; H, 6·64 %. $C_{31}H_{38}O_{11}$ requires C, 63·45; H, 6·53 %.) $[\alpha]_D^{25°}+57·1°$ ($c=0·0063$ in $CHCl_3$).

VII. *Methyl cholesterol-triacetylglucuronate.* Methyl acetobromoglucuronate (150 mg.) and cholesterol (146 mg.) were dissolved in benzene (8 ml.) and shaken overnight with 300 mg. of Ag_2CO_3. The product was worked up as usual and the resultant syrup crystallized spontaneously when rubbed with alcohol. The yield of crude product was 40 mg. (15 %). After three recrystallizations from absolute alcohol the substance melted at 162–164·5° after softening at about 152°. Yield of pure product: 18 mg. (about 7 %).

The substance crystallizes in fine platelets with a mother of pearl lustre. (Found: C, 68·06; H, 8·88 %. $C_{40}H_{62}O_{10}$ requires C, 68·33; H, 8·89 %.)

Biological activities of products

The following data may be given in the biological activities of the glucuronides.

I. A daily dose of 100 μg. of methyl dehydroandrosterone-triacetylglucuronate (containing 47·6 μg. of dehydroandrosterone) per capon, applied for 4 days dissolved in propylene glycol by smearing on the comb, was inactive.

II. A daily dose of 20 μg. of dehydroandrosterone-glucuronic acid (containing 12·4 μg. of dehydroandrosterone) per capon, applied for 4 days by smearing a solution in propylene glycol on the comb, was inactive. 77 μg. (containing 47·7 μg. of dehydroandrosterone) gave an average comb growth after 5 days of 14 %.

III. A daily dose of 50 μg. of methyl testosterone-triacetylglucuronate (containing 22 μg. of testosterone), applied to each capon in the same way for 4 days, was inactive.

IV. 12 μg. of methyl α-oestradiol-3-benzoate-17-triacetylglucuronate (containing 6·5 μg. of α-oestradiol-3-benzoate) were inactive in castrated mice. 60 μg. (containing 32·6 μg. of α-oestradiol-3-benzoate) produced oestrus when injected subcutaneously during 24 hr. in three lots of 20 μg. each.

V. 12 μg. of α-oestradiol-17-glucuronic acid (containing 6·8 μg. of α-oestradiol) were inactive. 60 μg. (containing 34·3 μg. of α-oestradiol), injected during 24 hr. in three lots of 20 μg., produced oestrus.

VI. 30 μg. of methyl oestrone-triacetylglucuronate (containing 13·8 μg. of oestrone), injected subcutaneously in 24 hr. in three lots of 10 μg., produced oestrus.

REFERENCES

Cohen & Marrian (1936). *Biochem. J.* **30**, 57.
—— —— & Odell (1936). *Biochem. J.* **30**, 2250.
Goebel & Babers (1935). *J. biol. Chem.* **110**, 707.
Venning & Browne (1936). *Proc. Soc. exp. Biol., N.Y.*, **34**, 792.

L. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES

VI. THE EFFECT OF REDUCING THE SALT CONTENT OF THE MEDIUM SURROUNDING THE CELL

By HUGH DAVSON[1]

From the Biological Laboratory, Cold Spring Harbor, and the Department of Physiology, University College, London

(*Received 16 January 1939*)

The effect of diminishing the electrolyte content of the medium bathing the cell, keeping the osmotic pressure constant, is generally recognized as an injurious one, whereby the normal selective permeability of the plasma membrane is disturbed; as an example of this the work of McCutcheon & Lucke [1928], who found that in glucose solution the permeability of *Arbacia* eggs to water is doubled as compared with eggs in sea water, may be quoted. In earlier papers [Davson, 1934; 1936; 1937; Davson & Danielli, 1936; 1938] some of the conditions which determine the erythrocyte's normal impermeability to cations, and changes in these conditions which lead to a breakdown of this impermeability, have been described. The material presented here is an extension of the work to the special case of the effect of an absolute decrease in the quantity of electrolytes in the suspension medium.

Joel [1915] has shown that if the erythrocytes of the ox are washed repeatedly with sugar solution and then suspended in the same medium, the latter shows an increase in electrical conductivity with time which is due, as he supposed, to the passage of salts from the cells; however, this treatment is drastic and there is no evidence that the changes observed were reversible and were not due to haemolysis. Jacobs & Parpart [1933], working under less extreme conditions, found changes in the fragility of the same erythrocytes on exposure to hypotonic solutions of non-electrolytes which they interpreted as being due partly to anionic exchanges and partly to the loss of cations from the cells. Maizels [1935] found, by direct chemical analyses, that the human erythrocyte loses K in glucose solution in a reproducible manner, the effect increasing with temperature.

The results of Joel and of Jacobs & Parpart are essentially indirect and, whilst the constructions placed upon them appear quite probable, experience has shown [Davson, 1936; 1937; Davson & Ponder, 1938] that indirect measurements as in conductivity and fragility studies are of questionable value in regard even to qualitative estimates of changes in cation permeability, whilst quantitative measurements are virtually excluded. Thus the results of Maizels remain as certain facts, inadequate in themselves, however, to allow of any explanation of the phenomenon of the induced cation permeability due to the reduction in the electrolyte content of the medium. Before entering into the experimental part of this paper, possible mechanisms for the cation permeability and the kinetics of the loss of K may be briefly touched upon.

[1] Beit Memorial Research Fellow.

If an erythrocyte is placed in a non-electrolyte solution there will, as Jacobs & Parpart [1933] have shown, be a tendency for the Cl^- and HCO_3^- to leave the cell; this loss of anions will be replaced by OH^- from the non-electrolyte medium. Thus, owing to the rapid shift of anions across the membrane, there will be a difference of *p*H (which may amount to three units if the dilution of the blood is great enough), the cells being alkaline in respect to the suspension medium. What seems not to have been realized hitherto is that in consequence of this *p*H difference there will be a large potential difference between the inside and the outside of the cell, the latter being negative in respect to the former. The magnitude of this P.D. will be given by the formula:

$$E = RT/F \ln [H_0]/[H_i],$$

where $[H_0]$, $[H_i]$ are the respective H ion concentrations inside and outside the cell, and thus may be of the order of 150 mV. As a result it would not be surprising if positive ions, should they come under the influence of this P.D., were repelled from the inside to the outside of the cell so that the normal restraint on these ions, which inhibits their migration, is overcome. Thus the phenomenon of cation permeability might reasonably be expected on suspending erythrocytes in a non-electrolyte medium. As K escapes from the cell it will be accompanied by Cl^- and HCO_3^- and, to a lesser extent, by OH^-; as a result of the migration of the HCO_3^- and OH^- ions the difference of *p*H tends to be reversed, and also the sign of the P.D., as the loss of K proceeds. If the cation permeability is due entirely to the acceleration of the ions down a potential gradient, then it must cease as soon as the difference in *p*H is neutralized; furthermore, the addition of salts at any time during the escape of K, in so far as it reverses the P.D. caused by the shift of anions, should inhibit the further loss of K.

If, however, the cause of the cation permeability is not the electrostatic acceleration of the ions but is due to the potential difference acting directly on the membrane, then the inhibition of the cation permeability due to the levelling out of the P.D. is only to be expected if the change in the membrane is a reversible one. If, further, it can be shown that addition of salts inhibits the migration of K after it has begun, and also that the latter process does *not* cease when the P.D. is neutralized or reversed in sign, it follows that the cation permeability is not due to the P.D. at all, and its cause must be sought in an actual change in the membrane due to the reduction in the salt content of the medium in contact with its exterior. The experimental work to be described may thus be considered in respect to these three general theories and it will be seen that by a process of exclusion one must adopt the last-mentioned in explaining the permeability to cations in non-electrolyte media.

The rate of loss of K will be given by the simple unimolecular equation:

$$\frac{dK_i}{dt} = k\,(K_i - K_0 - \beta),$$

where k is the permeability constant,[1] A the area of the cells, K_i and K_0 the concentrations of K inside and outside the cells respectively and the constant β equals the difference between the concentrations of K inside and outside the

[1] The use of a permeability constant for a single ion is not strictly correct, since the rate of permeation of a given ion will be a function of two probabilities, viz. those that a positive ion and a negative ion will pass the membrane at any given moment. However, in the case of the erythrocyte, which is so freely permeable to Cl^- and HCO_3^-, the use of a single constant for K^+ is probably justified, at least for the purposes of this paper. It will be seen that the experimental results deviate so greatly from the equation that this consideration is a minor one compared with the auto-inhibitory effect of the escape of K *per se*.

cells at equilibrium and is introduced into the equation to satisfy the condition that dK_i/dt equals zero when the K concentration inside the cell has dropped to its equilibrium value, which will be determined by the Donnan equilibrium, and will be greater than the concentration in the suspension medium; its absolute value will be small compared with the initial value of K_i owing to the large volume of suspension medium. *The equation assumes that the conditions determining the escape of K are unaffected by this escape.*

Now to a first approximation K_i, the concentration of K inside the cells, will be constant, since the loss of K plus its accompanying anions will result in a decrease in the osmotic pressure inside the cell causing the passage of H_2O into the suspension medium.[1] Under the conditions of the experiments K_0 may also be treated as a constant, and, owing to the disk shape of the cells, A will be approximately constant. Hence the equation becomes:

$$dK_i/dt = \text{Constant},$$

so that the loss of K should proceed linearly with time, and any gross deviation from linearity should be ascribed to a variation in k, the permeability constant, i.e. to a change in the properties of the membrane or the P.D. across it due to the loss of K.

Experimental

The non-electrolyte solution used was 0·32 *M* glucose, in glass-distilled water, for all species except human for which 0·32 *M* sucrose was used, since human erythrocytes are comparatively permeable to glucose. The whole blood was added to the non-electrolyte solution, contained in a centrifuge tube immersed in a thermostat at 25°; the ratio of the volumes of blood to suspension medium was 1 : 10. After the appropriate interval the suspension was centrifuged, the supernatant fluid was removed and the K or Na content of the cells determined as described earlier [Davson, 1934; 1937] using the method of Kramer [1920] for K and that of Barber & Kolthoff [1928] for Na. If the cells contained only a little K, as in the case of the cat, the dissolved ash was not made up to a definite volume from which aliquots could be taken but was evaporated to dryness in a silica beaker and the whole of the K in it determined; this procedure was also used for all Na determinations. The errors arising out of all steps in the procedure rarely amounted to more than 1%. As controls, cells suspended in non-electrolyte medium and immediately centrifuged down were used so that losses due to the centrifuging of the cells *per se* were discounted. *p*H changes in the suspension medium were determined colorimetrically.

Results

In Table I the variation in the K contents of the erythrocytes of the man, guinea-pig, rat, pig and rabbit with time of suspension in a non-electrolyte medium are shown; figures for three individuals of each species are presented to give some idea of the variability in the magnitude of the changes. The results are expressed as percentages of the control value. In Table II similar figures are given for the K and Na contents of the erythrocytes of the ox and cat; these

[1] Since about one-third of the K in the cells is in the form of a polyvalent haemoglobin salt, K_nHb, there will be an excess of osmotically active cations over anions, so that the loss of a pair of ions ($K + Cl^-$) or ($K + HCO_3^-$) reduces the osmotic pressure inside the cells by a greater amount than the fractional decrease in the K content. Hence, strictly speaking, the concentration of K should rise in the cells, although the amount per cell falls. This effect is compensated, however, by the increasing acidity inside the cell which reduces the amount of K_nHb.

Table I. *The variation of the K content of the erythrocytes of different species with time of suspension in a non-electrolyte medium. Temp. 25°*

	Man			Guinea-pig			Rat			Pig			Rabbit		
Time	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
Control	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1 hr.	74	80	82	84	91	98	95	93	92	99	—	99	98	100	101
2 hr.	57	69	69	76	79	94	89	87	86	99	99	99	98	99	100
3 hr.	52	60	59	69	74	93	86	83	—	93	—	99	98	99	96
6 hr.	—	—	—	—	—	—	—	—	—	—	96	98	—	—	—

Table II. *The variation of the K and Na contents of the erythrocytes of the ox and cat with time of suspension in a non-electrolyte medium. Temp. 25°*

	Ox						Cat					
	I		II		III		I		II		III	
Time	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na
Control	100	100	100	100	100	100	100	100	100	100	100	100
1 hr.	95	98	95	96	100	100	99	98	94	97	95	92
2 hr.	95	98	92	91	97	98	92	85	88	94	87	71
3 hr.	92	95	91	90	93	96	89	66	87	90	78	34
5 hr.	70	77	—	—	—	—	—	—	—	—	—	—

species differ from those in Table I in that their erythrocytes contain more Na than K. From Table I it is evident that the erythrocytes of the man, guinea-pig and rat show a well-defined permeability to K when suspended in a non-electrolyte medium; the cells of the pig and rabbit, however, are sharply differentiated from the former group in that the loss of K in the first 3 hr. is very small and of the order of 1–2 %. This species difference will be taken up later.

With regard to the results in Table II it may be remarked that the cells of the ox and cat show irregularities in their behaviour which were not encountered with the species in Table I. Thus in Exp. 1 of the ox there is a loss of 5 % in the K content during the first hour, no loss during the second hour, a loss of 3 % during the third, and of 22 % during the last 2 hr.; the same anomalous course was followed by the Na values; again in Exp. 1 of the cat we have a loss of 1 % of the K during the first hour, 8 % during the second hour and 3 % during the third hour. Another sort of irregularity occurs most markedly with the cat erythrocytes; this is in respect of the relative rates of loss of Na and K. It is generally realized that although K has a larger atomic weight than Na, the size of the K ion in aqueous solution is smaller than that of the Na ion in virtue of the greater hydration of the latter; consequently, if size determines the relative rates of migration of the two ions across the membrane, K should penetrate more rapidly than Na. This appears to be the case with the ox, and on occasions with the cat, but Exps. 1 and 3 for the cat show a much greater percentage loss of Na than of K during the intervals 1, 2 and 3 hr. That these irregularities can be due to experimental errors is quite out of the question, so that it would appear that under certain conditions Na may penetrate a membrane more rapidly than K, unless, of course, the equilibrium conditions determining the cessation of the permeability process are more complicated than would generally be expected; thus if the K in the cells is, at the outset, much nearer its equilibrium value than the Na, then comparisons between the losses of K and Na in given times would not necessarily lead to the determination of comparative permeability constants. The acceleration in the rate of loss of cations with time mentioned above will be discussed more fully later.

To return to the behaviour of some of the species in Table I, the K contents of the human, guinea-pig and rat cells are plotted against time of suspension in a non-electrolyte medium in Fig. 1; it will be seen from Table I that the curves are probably typical of the species, the rate of loss being in the order: man > guinea-pig > rat. The curves show deviations from linearity, the deviation being more pronounced and showing itself sooner the greater the initial rate of

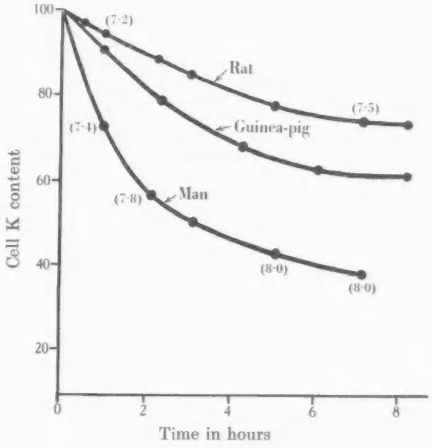


Fig. 1. Loss of K from the erythrocytes of the rat, guinea-pig and man in non-electrolyte solution at 25°. Ordinates: cell K content as a percentage of the control value. Abscissae: time of suspension in the non-electrolyte medium in hours. Figures in parentheses indicate the *p*H of the suspension medium after centrifuging down the cells.

loss. Furthermore, the curves tend to different stationary states; thus the rat cells tend to a value of 72·5, the guinea-pig to one of 60 and the human to 35 or less, these figures being, as before, percentages of the initial cell K contents. The figures near points on the curves are the values for the *p*H of the suspension medium after centrifuging and it is clear that the change over from an acid medium to an alkaline one mentioned in the introduction certainly occurs. However, the loss of K does not cease as soon as the medium becomes alkaline.

The reversibility of the induced permeability to K is brought out by the results of Table III and Fig. 2. In Table III, row (*a*) gives the loss of K during 1 hr. of suspension in the non-electrolyte medium followed by 2 hr. of suspension in the same medium to which NaCl had been added to bring the concentration of the latter up to 0·10 *M*. Row (*b*) gives the loss of K during 1 hr. of suspension in the non-electrolyte medium, and row (*c*) the loss during 2 hr. suspension in non-electrolyte solution plus NaCl. If the process is reversible (*b*) plus (*c*) should be equal to or greater than (*a*), and this is the case within the limits of experimental error. In Fig. 2, the curve *AB* gives the loss of human cells with time in sucrose solution; *AC* represents the loss in a sucrose plus NaCl solution (0·10 *M* with respect to NaCl). At the points *X* NaCl was added as before and the lines *XX'* show the loss of K subsequent to the addition of NaCl. The approximate parallelism of the lines again indicates reversibility, even after 2 hr. in the non-electrolyte medium alone. The slight loss of K from the cells in sucrose plus

Table III. *Experiments designed to show the reversibility of the induced permeability to K. (N.E. is abbreviation for non-electrolyte solution)*

Treatment	% loss of K		
	Man	Guinea-pig	Rat
(*a*) 1 hr. in N.E.; 2 hr. in N.E. + NaCl	20	23	13
(*b*) 1 hr. in N.E.	20	24	13
(*c*) 2 hr. in N.E. + NaCl	2	3	0
(*b*) + (*c*)	22	27	13

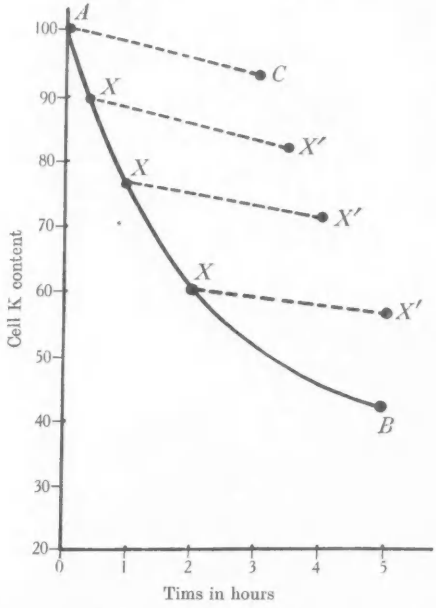


Fig. 2. Curves demonstrating the reversibility of the induced permeability to K. Temp. 25°.

NaCl indicated by the line AC is not due to the presence of sucrose since it occurs in isotonic NaCl alone [Maizels, 1935; Davson, 1937].

In Fig. 3 the loss of K from the human erythrocyte in 2 hr. is shown as a function of the electrolyte content of the medium and it is seen that the effect of diminishing the electrolyte content of the medium begins when the former has been reduced to less than 0·09 M. The extreme sensitivity of the rate of loss to salt content of the suspension medium at values of the latter below 0·016 M is another interesting point.

From the curves in Fig. 1, the coincidence of the retardation of the rate of loss of K with the increasing alkalinity of the suspension medium might lead one to suppose that the cause of this retardation was the alkalinity; this, however, would be in contradiction to Maizels' results which showed an increased rate of loss in an alkaline medium, although too much weight must not be placed on these results since the alkalinity was produced by addition of NaOH to the medium giving an initial reaction of the medium of about pH 10 which in itself is sufficient

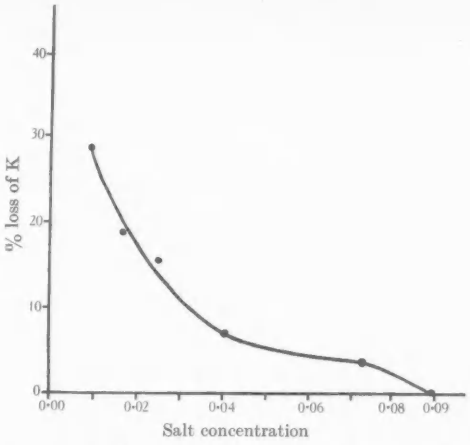


Fig. 3. Variation in the rate of loss of K from human erythrocytes with salt content of the suspension medium. Ordinates: percentage loss of K in 2 hr. Abscissae: concentration of NaCl in the suspension medium in moles/litre. Temp. 25°.

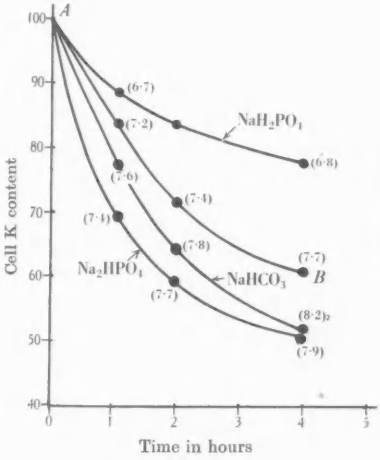


Fig. 4.

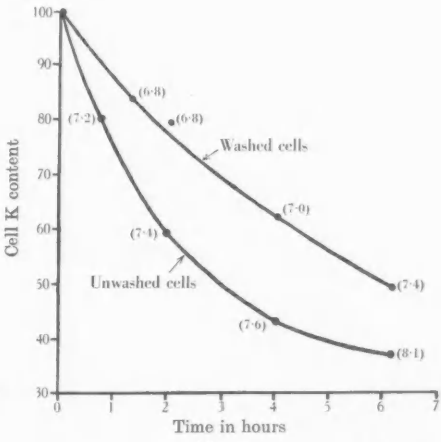


Fig. 5.

Fig. 4. The effect of the addition of acid and alkaline buffers to the non-electrolyte medium on the loss of K from human cells. *AB* is the control curve for non-electrolyte medium plus NaCl. Temp. 25°.

Fig. 5. Comparison of the losses of K from washed and unwashed human cells in non-electrolyte media. Temp. 25°.

to produce irreversible damage. In Fig. 4 is shown the effect of the addition of the buffers NaH_2PO_4, $NaHCO_3$ and Na_2HPO_4 to the non-electrolyte medium on the loss of K from human cells. The control curve, *AB*, represents the loss of K in sucrose solution to which NaCl had been added to make the total ionic con-

centration equal to that in the test solutions to which the buffers had been added (0·008 *M*). The effect of the buffers may, therefore, fairly be attributed to the *p*H changes they produce. The curves show that alkaline buffers accelerate the rate of loss of K, thus confirming Maizels' conclusions, whilst the acid buffer NaH_2PO_4 retards it. The cause for the falling off in the rate of loss with time must therefore be sought elsewhere.

In Fig. 5 the curves for the loss of K from both unwashed and washed human cells are compared. In this experiment a portion of the blood was centrifuged in a graduated tube, the serum was removed and replaced with an equal quantity of Ringer solution containing Na, K and Ca in the proportions normally present in mammalian serum, without, however, any bicarbonate. After mixing the cells with the Ringer solution, the two samples of blood, normal and washed, were measured out in 1 ml. lots into sucrose solutions as before. The curves show that the unwashed cells lose K more rapidly than do the washed ones; on the other hand the normal slowing down of the rate with time hardly shows itself with the washed cells, so that with long times of suspension the discrepancies between the losses under the two conditions tend to become smaller instead of larger. It is to be noted that for points of equal loss of K, the suspension medium containing the unwashed cells is always more alkaline, and the difference in the rates of loss may, in conformity with the curves in Fig. 4, be ascribed to the difference in the *p*H of the suspension media, that containing the unwashed cells being more alkaline because of its greater bicarbonate content. It will be seen in the general discussion later that the absence of any gross deviation from linearity shown by the washed cells may be closely connected with the removal of the bicarbonate from the cells during the washing.

Table IV. *Erythrocytes of the rabbit. Temp. 25°*

Exp. no.	Treatment	Cell K content
1	Control	100
	2 hr. in isotonic glucose solution	99
	4 hr. ,, ,,	99
	5 hr. ,, ,,	98
	6 hr. ,, ,,	97
	7 hr. ,, ,,	96
2	Control	100
	Cells washed twice in glucose then suspended in glucose solution for 2 hr.	93
	,, ,, ,, ,, 4 hr.	85
	,, ,, ,, ,, 6 hr.	80
3	Control	100
	1 hr. in isotonic glucose solution	100
	1 hr. in isotonic glucose 5×10^{-5} *M* Cu	98

The results shown in Table IV may now be briefly enumerated. Exp. 1 shows that the erythrocytes of the rabbit maintain their virtual impermeability to K in glucose solution for as long as 7 hr. of suspension; there is indeed a loss of 4 % by the end of this time but the figures show that it is distributed evenly over the whole period. Washing the cells of the rabbit twice with glucose before suspension in glucose solution for a definite time causes a fair degree of permeability to K (Exp. 2). Addition of copper to the non-electrolyte medium causes a rapid sedimentation of the rabbit cells but does not produce any marked permeability to K.

Discussion

The theory that the permeability of the erythrocyte to cations, induced by the diminution of the electrolyte content of the suspension medium, is due simply to an electrostatic repulsion of K and Na by the potential gradient across the membrane must be abandoned, since the loss of cations does not cease when the suspension medium becomes more alkaline than the contents of the cell (in virtue of the buffering power of haemoglobin very little change in the *p*H of the inside of the cell will occur during the escape of K; any change which does occur will be to make the contents more acid). As the P.D. considered here is a thermodynamic one, depending on the relative concentrations of Cl^- and HCO_3^- in the cells and suspension medium, only small and irregular variations between species should occur in it. However, it is found that the rabbit and pig cells show little tendency to lose K under conditions where human cells lose as much as 70% in 7–8 hr. This again presents a difficulty in the way of interpreting the permeability as an electrostatic phenomenon, although it may be overcome by the assumption that the normal restraints on the migration of cations vary in strength among the species, being greatest with the rabbit and pig and least with the human cells. This assumption is not in accord, however, with previous work on species characteristics [Davson, 1937]. Passing, then, to the next possible mechanism, viz. that the P.D. acts on the membrane, possibly by a re-orientation of the dipole constituents of the membrane, then the loss of K must only be expected to cease when the P.D. is neutralized provided that the change in the membrane is reversible. Now it has been shown that the induced permeability to K is indeed reversible by addition of salts, so that it would, on this theory, be necessary that the permeability to K should cease as soon as the P.D. were neutralized. We must therefore reject the P.D. as a significant factor in causing the permeability to cations; hence the cause of the permeability must be sought in a reversible change in the membrane due to the decrease in the salt content *per se*, apart from any potential effects due to ionic exchanges across this membrane.

Attention should be drawn here to a correlation between the magnitude of the induced permeability to cations and the rate of sedimentation of the erythrocytes in the non-electrolyte solutions. The erythrocytes of all species generally sediment more rapidly when suspended in a non-electrolyte solution than in isotonic NaCl, but this difference is far more pronounced in the case of the human, guinea-pig, rat, ox and cat cells than with the rabbit and pig cells; in fact individual samples of pig blood have been obtained which showed no evidence of settling, when added to glucose solution, after an hour, whereas if human or guinea-pig blood is treated in the same way the cells drop to the bottom of the tube within 5 min. or less. The reason for the rapid sedimentation is, of course, the agglutination; the suspensions which sediment rapidly contain large aggregates of cells whereas a suspension of pig blood is found to contain only discrete cells.[1] Washing the rabbit cells, which normally show very little permeability to cations in a non-electrolyte medium, twice with glucose solution and then suspending them in a further quantity causes them to lose K fairly readily, and at the same time marked agglutination is observed. Thus there

[1] This correlation is not perfect; thus one specimen of rat blood gave no sign of sedimentation and yet lost K at a rate typical for this species. Similarly, a specimen of rabbit blood sedimented fairly rapidly with no loss of K; in this case, nevertheless, there was no observable agglutination. No specimen of human, guinea-pig, or cat blood has, however, been found which did not show marked agglutination.

seems to be some change in the surface of the erythrocyte, produced by the reduction of the electrolyte content of the medium, which allows agglutination to occur and at the same time induces a permeability to cations. That the agglutination itself does not cause the cation permeability but is only symptomatic of the changes which do produce it is shown by the fact that addition of Cu to the non-electrolyte medium, which causes rabbit cells to agglutinate to a tremendous extent, does not cause any marked loss of K; further, addition of acid phosphate to the medium decreases the rate at which K escapes from human cells whilst it increases the degree of agglutination. The theory that a change in the surface of the erythrocyte is responsible for the cation permeability would be in accord with the apparently anomalous behaviour of the ox cells; here we had an acceleration of the rate of loss as the time of suspension increased, and this could be accounted for by assuming that the change in state of the surface of the erythrocytes of this species takes time, so that during the first hour or so the cells will not be very permeable whereas later, when the change becomes more pronounced, the permeability increases.

To determine the nature of this surface change presents some difficulties. The effect of a reduction in the salt content of the medium on such systems as protein sols or thin films of lipoids has been studied and has been described as a "shift of the isoelectric point" [Michaelis & Rona, 1919] or a change of the "surface pH" [Danielli, 1937], and these descriptions agree in so far as the changes expected will be those occasioned by a shift of the pH of the medium to the acid side, if the dissociating groups in the systems are on the alkaline side of their isoelectric points. As the ionizing groups in the erythrocyte membrane are quite definitely on the alkaline side of their isoelectric point [see e.g. Abramson, 1934], if the phenomenon of the induced permeability to cations has any similarity with the changes described by Michaelis & Rona and by Danielli, then it would be expected that it would be inhibited by alkaline and accelerated by acid buffers, yet we have seen that the reverse is true; acid phosphate reduces the rate of loss of K far more than an equivalent quantity of a neutral salt such as NaCl, whilst alkaline phosphate or bicarbonate does not reduce the rate of loss as much as NaCl does. The change in the surface of the erythrocyte is, then, not one which could be simply ascribed to a change in the ionization of its acid groups, and it is interesting to note, if the correlation between the agglutination of the cells and their induced permeability to K means anything, that Abramson [1934] has shown that the charge on the surface of the erythrocyte, measured electrocataphoretically, is not the determining factor in rouleau formation and therefore, presumably, in agglutination. Further investigation of this point must be on a system which allows changes in the salt content of the medium being made independently of changes in the pH. With the intact erythrocyte this is impossible owing to the exchanges of anions. Such a system might be given by a spread film of the extracted lipoids from the erythrocyte [Gorter & Grendel, 1925] and work on this is under way [Danielli & Davson]; however, it may well be that the permeability to cations is a result of an asymmetry of the membrane, since the concentrations of salts inside and outside are, under the conditions of suspension in a non-electrolyte medium, widely different, so that studies on films may not throw light on the problem.

To return to the actual experimental data, there is a final point which requires attention, namely the falling off in the rate of loss of K with time, which occurs at quite an early stage in the process. The equation derived to suit the conditions of the experiments obviously does not fit the observed data, and it seems, in view of the large deviation from linearity, that the discrepancy is not

due to any of the approximations introduced in the derivation, but rather to the "permeability constant" being a function of the loss of K at any given moment. The alkalinity of the suspension medium caused by the migration of K is not the cause of this auto-inhibition since alkalinity favours the loss of K; similarly the increased salt content of the medium can have little influence owing to the large volume of the latter. A possible factor is the P.D. which will be built up by the migrating HCO_3^-; as the loss of K proceeds, the inside of the cells will become acid whilst the suspension medium becomes alkaline, and this difference in reaction will be reflected in a difference in potential, and this latter may eventually become great enough to inhibit the further loss of K. Support is given to this view by the curves of Fig. 5, in which the losses of K from cells, out of which most of the HCO_3^- has been washed, are compared with those from cells with their normal contents. It was seen that the washed cells showed only a very little inhibition of the loss of K after 6 hr. of suspension whilst the unwashed cells showed marked inhibition after 2 hr. suspension. This difference in behaviour may be attributed to the difference in the HCO_3^- content of the cells; the passage of a given quantity of K will be followed by a greater *p*H difference when this K is accompanied by HCO_3^- than when it is accompanied by Cl^-. It was noted that the *p*H values for the suspension media agreed with this view. Thus although the P.D. due to an excess of OH^- in the cells over that in the suspension medium is not the chief factor in causing the migration of K, it may be of importance in determining its cessation. The slower the initial rate of loss, i.e. the greater the obstacle offered by the membrane to the passage of K, the smaller will be the P.D. required to inhibit the loss; hence the loss of K from the rat cells will be expected to cease at a smaller external *p*H than that from human cells, and this is so.

Two practical points following from this work may be touched upon. Many studies of the permeability of erythrocytes to non-electrolytes are made by adding a small quantity of blood to a large volume of the pure non-electrolyte solution, and using the time required for haemolysis as an index to the rate of penetration of the non-electrolyte [*vide* e.g. Jacobs *et al.* 1935]. The customary dilution of the blood in the non-electrolyte medium is 1 in 500, and it is quite clear from the present work that even at a much lower dilution (1 in 10) the erythrocytes of many species are in a definitely abnormal state, so that the results of this type of work, interesting and important as they are, may nevertheless not represent the behaviour of cells in their normal environment. Jacobs *et al.* [1937] have indeed studied the influence of salts on the permeability of ox erythrocytes to glycerol, and have found changes which, however, can be at least partly, if not wholly, ascribed to changes in the volume of the cells due to anionic exchanges. It would be interesting if this work were extended to the erythrocytes of species which show cation permeability in a non-electrolyte medium more markedly, e.g. human and guinea-pig cells, since the demonstration of a normal behaviour in respect to the permeability to non-electrolytes and an abnormal behaviour regarding cation permeability might lead to important conclusions relating to the structure of the erythrocyte membrane.

A more disturbing point is the effect of the escape of K on the rate of haemolysis when this is being used as an index to the rate of penetration of a non-electrolyte. From the curve in Fig. 3 it follows that with a dilution of blood of 1 : 500 the rate of loss of K from the erythrocytes of some species may be many times greater than that described here; as a result of this loss of K the amount of non-electrolyte which must penetrate a cell to cause it to burst will be greater, so that the time required for haemolysis will be no true index to the rate

of penetration of the non-electrolyte. This will be especially true in respect to the determination of temperature coefficients, since the effect of a rise in temperature will be two-fold: (*a*) an increase in the rate of penetration of the non-electrolyte, and (*b*) an increased escape of K. These effects will be antagonistic, and it is interesting to note that Jacobs *et al.* [1935] find with certain species an actual decrease in the rate of haemolysis in glycerol solutions on raising the temperature, and this may be due to the fact that the influence of (*b*) outweighs that of (*a*). Probably this consideration does not affect the main conclusions drawn by Jacobs *et al.* from their measurements of temperature coefficients, but it would be a serious factor in any calculations of the kinetics of penetration in which values of the Q_{10} determined under these conditions are included.

Summary

The permeability of the erythrocytes of several species to cations, on suspending them in a non-electrolyte medium, has been investigated. The cells of the man, guinea-pig and rat are permeable to K, and those of the ox and cat to both Na and K, under these conditions; the latter species show irregularities in that there may be an acceleration of the rate of loss of cations with time, and in that Na may sometimes penetrate more rapidly than K. The cells of the man, guinea-pig and rat give smooth curves on plotting their K contents against time of suspension; however, there is a falling off with time in the rate of loss of K, so that a stationary state is reached at times remote from the equilibrium position. This auto-inhibition is ascribed tentatively to the building up of a potential gradient which eventually inhibits the further migration of K. The erythrocytes of the rabbit and pig show little tendency to lose K under the same conditions, the loss after 7 hr. of suspension may be as low as 4 % compared with a loss of nearly 70 % with human cells.

The change in the membrane causing the permeability is a reversible one in that the escape of K may be inhibited after it has begun by the addition of NaCl to the non-electrolyte medium.

Human cells are very sensitive to salt concentration of the suspension medium when this is less than 0·02 *M*; at a concentration of 0·09 *M* the non-electrolyte effect ceases.

Alkaline buffers added to the suspension medium reduce the rate of loss of K to a smaller extent than an equivalent quantity of NaCl; acid buffers have the reverse effect.

There is an apparent correlation between the tendency of the erythrocytes of different species to agglutinate in a non-electrolyte medium and the induced permeability to K; the agglutination itself is not the cause of the permeability to K, but may be symptomatic of a change in the surface of the cell which allows cations to cross the membrane.

The author wishes to thank Dr Eric Ponder for his invitation to work in his laboratory at Cold Spring Harbor, where part of this work was done, during the summer of 1938, and the Long Island Biological Association for defraying all personal expenses involved by his acceptance of this invitation.

REFERENCES

Abramson (1934). Electrokinetic Phenomena. New York.
Barber & Kolthoff (1928). *J. Amer. chem. Soc.* **50**, 1625.
Danielli (1937). *Proc. roy. Soc.* B, **122**, 155.
Davson (1934). *Biochem. J.* **28**, 676.
—— (1936). *Biochem. J.* **30**, 391.
—— (1937). *J. cell. comp. Physiol.* **10**, 247.
—— & Danielli (1936). *Biochem. J.* **30**, 316.
—— —— (1938). *Biochem. J.* **32**, 991.
—— & Ponder (1938). *Biochem. J.* **32**, 756.
Gorter & Grendel (1925). *J. exp. Med.* **41**, 439.
Jacobs, Glassman & Parpart (1935). *J. cell. comp. Physiol.* **7**, 197.
—— & Parpart (1933). *Biol. Bull. Wood's Hole*, **65**, 512.
—— —— & Corson (1937). *J. cell. comp. Physiol.* **9**, 177.
Joel (1915). *Pflüg. Arch. ges. Physiol.* **161**, 5.
Kramer (1920). *J. biol. Chem.* **85**, 47.
McCutcheon & Lucke (1928). *J. gen. Physiol.* **12**, 129.
Maizels (1935). *Biochem. J.* **29**, 1970.
Michaelis & Rona (1919). *Biochem. Z.* **94**, 225.